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The actin-driven spatiotemporal organization of T cell signaling at the system scale

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Running title

Organization of the T cell signaling system

Abstract

T cells activate in a cellular interaction with antigen presenting cells (APC). During activation receptors and signaling intermediates accumulate in diverse spatiotemporal distributions. These distributions control the probability of signaling interactions and thus govern information flow through the signaling system. Spatiotemporally resolved system scale investigation of signaling can extract the regulatory information thus encoded, allowing unique insight into the control of T cell function. Substantial technical challenges are briefly discussed. While much of the work assessing T cell spatiotemporal organization uses planar APC substitutes, we focus here on B cell APCs with often-stark differences. Spatiotemporal signaling distributions are driven by cell biologically distinct structures, a large protein assembly at the interface center, a large invagination, the actin-supported interface periphery as extended by smaller individual lamella, and a newly discovered whole-interface actin-driven lamellum. The more than 60 elements of T cell activation studied to date are dynamically distributed between these structures generating a complex organization of the signaling system. Signal initiation and core signaling prefer the interface center, while signal amplification is localized in the transient lamellum. Actin dynamics control signaling distributions through regulation of the underlying structures and drive a highly undulating T cell/APC interface that imposes substantial constraints on T cell organization. We suggest that the regulation of actin dynamics, by controlling signaling distributions and membrane topology, is an important rheostat of T cell signaling.

Keywords

T cell signaling, systems biology, imaging, actin

Introduction

T cells activate in a cellular interaction with antigen presenting cells (APC). Ever since the ground-breaking discoveries by the Kupfer laboratory (1, 2), it is well established that receptors and signaling intermediates are not evenly distributed across an activating T cell but enrich at particular locations at particular times, referred to here as ‘spatiotemporal patterning’. Interface recruitment of receptors and signaling intermediates has since been described for dozens of other proteins and lipids (3-15), yet a comprehensive understanding of the function of such uneven signaling distributions has remained elusive. Considering first principles, in live cells enrichment of two proteins at the same time and location as mediated by spatiotemporal patterning increases the interaction probability of the co-localized signaling intermediates. Across many signaling interactions, spatiotemporal organization thus determines how regulatory information flows through signaling networks in time and space. Because of the complexity of signaling, this regulatory information only becomes evident at the system scale. A comprehensive understanding of how a signaling system is organized in time and space thus should in principle reveal how signaling information is processed during cellular activation and provide unique access to mechanisms of the regulation of cellular function.

Here we discuss experimental constraints in studying the spatiotemporal organization of T cell signaling, some of the principal features of spatiotemporal patterning in the activation of T cells by B cell APCs, the role of actin dynamics in driving the spatiotemporal organization, and initial experiments to generate causality by experimentally manipulating the spatiotemporal organization of T cell signaling. Uneven subcellular distributions also occur in other aspects of T cell biology, e.g. in T cell migration (16, 17) and during asymmetric cell division (18, 19) as well as in virtually every other cell type (20-22). While we will focus on T cell activation here, principles of how the spatiotemporal organization of signaling governs cellular function are likely broadly applicable to cell signaling.

Studying the spatiotemporal organization of lymphocyte signaling

Image analysis

A system scale investigation of the spatiotemporal organization of T cell signaling requires the acquisition of large amounts of imaging data. To account for single cell variability, a substantial number of individual T cell:APC couples must be analyzed for each signaling intermediate studied, preferably 50 or more. Thus even a modest study of the system scale organization of T cell signaling, e.g. using twenty sensors across a few experimental conditions, necessitates the acquisition and analysis of imaging data on thousands of T cell:APC couples. These images are rich in information: they describe the concentration of signaling molecules within each region of a T cell and how these concentrations change during activation by an APC. Analysis of these four-dimensional maps of the local concentrations of the signaling sensor are complicated by a number of factors that require advanced image analysis approaches that are only in the early stages of development. These factors include the movement of cell couples during acquisition of movies, changes in cell shapes over time, variation in

the morphology of the T cell:APC interface, and, most importantly, significant variation in the “maps” of the same sensor from cell to cell. Since the goal is not simply automated recognition of different subcellular patterns (as has been extensively described for other cell types (23)), general-purpose image analysis packages widely used for cell screening (reviewed in (24)) are not sufficient. The goal is instead to reconstruct the relationships between different sensors measured in different sets of cells *as if they had all been measured in the same cell*. The variation in patterns from cell to cell means that this cannot be accomplished by simply superimposing the “maps” of different cells. Instead, it is necessary to construct models of each sensor distribution that correct for changes in cell shape and appropriately capture cell-to-cell variation, and then register these models to each other. A first required step is segmentation, i.e. the computational identification, of individual T cells. The segmentation of T cells in fluorescence images with the subsequent aim of measuring changes in T cell polarity or to automatically generate unbiased pattern classifications have been described (25, 26)(B. H. Cho, C. Wülfing and R. F. Murphy, unpublished data). Once identified, T cell:APC couples must be tracked over time, and then variation in cell shapes both for the same cell over time and between different cells must be addressed. One approach for this is to use non-parametric deformation-based morphometry methods, such have been used to characterize nuclear shape changes (27). Lastly, approaches will be needed to build generative models of the protein distributions, as has been done for various structures in other cell types (28). Ultimately, the registered spatiotemporal models of protein distribution can be used in cell simulations to provide a detailed understanding of the interactions and sequence of events during T cell activation. Initial work on modeling the interactions between fairly modest numbers of proteins within the spatiotemporal constraints of a cell couple using Simmune have been described (29), and a number of other systems for spatially-resolved cell simulation, such as Virtual Cell, MCell, and Smoldyn, are also available (they are extensively described on their websites).

Currently, traditional cell biological pattern classification approaches, i.e. the percentage of cell couples displaying a stereotypical spatiotemporal phenotype, most often have to substitute for a comprehensive quantitative analysis. As long as the classification used reflects critical elements of the spatiotemporal organization of T cell signaling, useful data can emerge. For example, we use seven patterns defined by intensity and geometrical constraints to describe spatiotemporal distributions in T cell activation (30). ‘Distal’ accumulation reflects accumulation away from the interface at the distal pole complex (31) and is often excluded from analysis when the focus is on T cell:APC interface distributions. Six mutually exclusive patterns classify sensor accumulation at the interface (Fig. 1A). ‘Peripheral’ accumulation in a ring around the edge of the interface, the Kupfer pSMAC (2), reflects an area rich in F-actin polymerization (32, 33), ‘asymmetric’ accumulation represents smaller individual actin protrusions. ‘Central’ accumulation, the Kupfer cSMAC, covers a large signaling complex at the interface center. Accumulation in a transient often μm -deep ‘invagination’ has been tentatively linked to early resetting of T cell signaling (34). Newly discovered accumulation in a transient, deep, and wide F-actin-supported lamellum (‘lamellar’) is discussed extensively below. Only the sixth pattern, a ‘diffuse’ accumulation covering the entire interface and localized to our best approximation at the plasma membrane at this time lacks a more specific cell biological foundation and mostly captures interface accumulation without well-developed spatiotemporal features.

T cell activation for imaging

T cells function in vivo. Nevertheless, principal features of T cell:APC coupling are well conserved in vitro: individual or sequential tight cell couples form such that the frequency of formation and the cell couple duration are dependent on the strength of stimulus (35-38). Interestingly, T cell coupling in collagen-rich three-dimensional environments becomes substantially more transient (39). Principles of spatiotemporal patterning described in vitro thus likely hold up in secondary lymphoid organs but not necessarily in other in vivo environments.

T cells are small cells (7 – 9 μm) that activate in a cellular interaction with APCs. T cell/APC couples are uniquely difficult to address with cell biological approaches because of their small size and their three-dimensional nature. To circumvent these difficulties, a large part of the literature addresses the organization of T cell signaling using planar APC substitutes, supported lipid bilayers or antibody-coated cover slips. Such APC substitutes allow for live cell imaging in the single plane of the bilayer or antibody-coated coverslip, thus enabling single molecular tracking and selective imaging of the plasma membrane and its immediate vicinity (11, 32, 40-42). Among many critical insights thus gained is that receptors and signaling intermediates form microclusters of dozens of molecules at the interface periphery and then move to the interface center. APC substitutes also allow almost complete control over the composition and mobility of the activating surface. This has been elegantly used to count the number of TCR ligands that need to be engaged in individual TCR clusters (43). With respect to the investigation of the spatiotemporal organization of the T cell signaling system it is generally assumed that the planar T cell:APC substitute interface constitutes a reasonable approximation of an actual T cell:APC interface. Recent work on T cell membrane topology and its underlying actin dynamics as discussed below suggests that this assumption is at best partially correct. In the interpretation of data from studies on the organization of T cell signaling it therefore needs to be taken into account, how differences in membrane topology and molecular composition (see section on sensitivity to activation conditions below) between planar APC substitutes and actual APCs drastically affect signaling organization.

The spatiotemporal organization of lymphocyte signaling is complex and sensitive to activation conditions

The spatiotemporal organization of lymphocyte signaling is complex

The spatiotemporal organization of T cell signaling at the system scale is highly complex. Within a set of 54 signaling sensors that show accumulation at the T cell/APC interface it is virtually impossible to find two sensors whose pattern classification into the six interface patterns introduced above cannot be distinguished with statistical significance (Fig. 1). Even small changes in the overall spatiotemporal organization of T cell signaling will thus differentially affect a large number of signaling interactions. The extent of protein redistribution in spatiotemporal patterning is modest (1, 30) leading to a ~ 3 -fold enrichment in the concentration of the sensor in the area of accumulation and to little change in its concentration in the

remainder of the cell. Because of the high diversity and modest extent of spatiotemporal distributions, cellular function can be efficiently modulated by changes in spatiotemporal patterning but not switched ‘ON’ or ‘OFF’. Technically, because of the diversity in spatiotemporal distributions the localization of an individual signaling intermediate contains limited information outside the system context. Analyzed within the system context however, such localization can provide powerful functional hypotheses: By determining which cell biological structures and molecules with known function the protein of interest is closely associated with in time and space, its function can be inferred. For example, the Basilic mutation in Rltpr that phenocopies CD28-deficiency was implied in receptor internalization through a shift in Rltpr-Basilic localization relative to that of the wild type Rltpr protein from signaling- to internalization-associated spatiotemporal patterns (44).

The spatiotemporal organization of lymphocyte signaling is sensitive to activation conditions

As the spatiotemporal organization of signaling is a sensitive regulator of the information flow through the T cell signaling system, it is of interest to understand how it differs under diverse T cell activation conditions. While comparisons of the spatiotemporal organization of T cell signaling across large numbers of imaging sensors are largely missing, 16 sensors compared across two experimental conditions is the most we are aware of (45), even the comparison of individual sensors or small groups thereof across different T cell activation conditions strongly suggests that the spatiotemporal organization of T cell signaling is highly sensitive to T cell activation conditions. In T cell differentiation, thymocytes undergoing positive and negative selection display hardly any or distinctly less patterning (46, 47), Th2-polarized T cells showed less central TCR patterning and lipid raft accumulation than Th0 or Th1 cells (45, 48), regulatory T cells had PKC θ shifted from the T cell:APC interface to the distal pole (49), and interface accumulation of LAT and Vav were more transient in CD8 versus CD4 T cells (50, 51). Activation of T cells with self-reactive TCRs yields less pronounced spatiotemporal organization (52). B7 blockade made sensor accumulation more transient, ICAM blockade shifted the balance between central and peripheral accumulation, and lower agonist peptide doses strongly affected signaling centrality (30). A lower affinity peptide impaired accumulation at the interface center (1). In our own work, using four sensors to compare the activation of primary OTII T cells by primary B cell blasts and Ova wild type peptide to that with a newly developed partial agonist, Ova E336Q, overall interface accumulation was diminished with the OvaE336Q variant and accumulation was shifted from patterns driven by defined cell biological structures (central, invagination, peripheral, lamellar – see below) to diffuse accumulation and enrichment in individual smaller actin-driven protrusions (P.S. and C.W., unpublished). A comparison of data on the spatiotemporal organization of T cell signaling across different T cell activation conditions thus has to be executed with great care.

Despite this variability in response to different stimuli, the spatiotemporal organization of T cell signaling seems well conserved when T cells expressing different TCRs are compared upon similar stimulation. In the absence of system scale studies, we compared interface accumulation of PKC θ or Vav, markers with strong central and lamellar/peripheral perence, in the activation of 5C.C7 (53), DO11.10 (54), OTII (55), and AND (56) primary T cells with B cells and a high concentration

of agonist peptide. Spatiotemporal preferences were well preserved even when the overall extent of interface accumulation varied moderately (Fig. 2). A comparison of spatiotemporal patterning with 20 sensors between 5C.C7 and DO11.10 T cells corroborates such similarity (Roybal et al., unpublished data). While sensitive to T cell activation conditions, the spatiotemporal organization of T cell signaling does seem robust across T cells expressing different TCRs.

T cell signaling segregates dynamically between a stable interface center and a transient actin-supported lamellum

The determination of the spatiotemporal distributions of 54 elements of T cell activation at the T cell:APC interface allows us to characterize the flow of signaling information in time and space during early T cell activation. Patterning data and representative videos are available at www.bristol.ac.uk/cellmolmed/research/infect-immune/wuelfing/. The first ten minutes of T cell activation are of particular interest as they contain the most dramatic changes in cell morphology, the peak of biochemically detectable T cell signaling, and the translocation of key transcription factors such as NFAT and NF κ B to the nucleus (30, 57-59). In the activation of primary T cells by B cells in the presence of a high concentration of agonist peptide, ligand engaged receptors, the TCR, CD2, and CD28 accumulate at the center of the T cell:APC interface within seconds of the formation of a tight cellular interface (Fig. 3A). The TCR often transits through the distal pole complex on its way to the interface. Even ligand-engaged LFA-1 initially accumulates at the interface center, yet moves to the interface periphery within a few minutes. The center of the interface thus is the site of preferential receptor engagement. The most proximal TCR signaling intermediates, Lck, ZAP-70, and LAT, the latter both as total protein and phosphorylated at Y191, similarly are enriched at the interface center (Fig. 3B). This suggests that the ligand-engaged receptors at the interface center are involved in active signal transduction. In contrast, a large and diverse group of more distal signaling intermediates dynamically localizes to a large actin-supported lamellum (Fig. 3C), further characterized in the next section of this review. Lamellar signaling intermediates include the phosphatidylinositol lipid PIP₂, the Rho GTPase family guanine nucleotide exchange factor Vav1, the phosphatases SHP-1 and Chronophin, the mediators of LFA-1 avidity regulation SKAP55 and ADAP, the transcription factor NF κ B prior to nuclear translocation, the motor protein Myosin 1C, and the regulator of thymic selection Themis (60). The preferred localization of a substantial part of T cell signal transduction thus is distinct from that of signal initiation. Interestingly, a number of adaptor proteins dynamically connect the interface center to lamellar signaling, as they transition from an immediate but brief central accumulation to a lamellar localization (Fig. 3D). This transition is most pronounced for SLP-76 where 97 ± 3 % of T cells with initial central accumulation display lamellar patterning at a later time point. Grb2 and Nck also move from the center to the lamellum, even though a substantial percentage of cell couples retain sustained central accumulation, indicative of an only partial transition. Other prominent T cell signaling intermediates, in particular PLC γ and PKC θ , display sustained accumulation at the interface center. PLC γ is likely active there as one of the PIP₂ cleavage products, diacylglycerol (DAG) is also centrally localized (Fig. 3E). Given the central role of PIP₂ cleavage in T cell signaling, these data suggest that the T cell core signaling machinery is stably enriched at the interface center. While central

localization of receptors and signaling intermediates is fairly well sustained, lamellal patterning is transient (Fig. 3C and see below). Interestingly, some signaling intermediates such as CD2AP and PI 3-kinase transition from lamellal accumulation to the interface center upon dissolution of the large T cell lamellum (Fig. 3F). Lamellal signaling is thus connected to the interface center both upon its initiation and its termination. The system scale signaling distributions thus characterize the interface center as the preferred location of signal initiation and core signal transduction, while a transient actin-supported lamellum hosts many processes related to signal modulation and amplification (Fig. 3G). Once we have summarized the biophysical characteristics of central and lamellal signaling in the next section, we will discuss potential rationales for signaling segregation.

Using a T cell line rather than primary T cells, fixed cell couples staining rather than live cell imaging, and a smaller set of sensors, Freiberg et al. have previously identified a comparable bi-directional signaling transition (61). Based on representative images shown in these studies, T cell signaling as determined with antibodies against phosphotyrosine, Lck, and phosphorylated ZAP-70 was initiated in less than a minute covering the entire interface, similar to the rapid kinetics of signaling in live cells. Subsequently, signaling segregated into a central area (Lck) and an area surrounding the center (phosphotyrosine), similar to central and lamellal signaling, before it became more homogeneous again. Interestingly however, Freiberg et al. found a transient dip in signaling activity at the interface center at the peak of segregated signaling. Not covered by our sensor set, transient recruitment of CD45 to the interface center was suggested to play a major role in the segregation of signaling and its transient decrease in intensity. The transient dip in signaling intensity was not evident in the live cell imaging data. Possibly signaling complexes were smaller while moving from one pattern to another and thus more difficult to fix for the staining experiments.

Actin drives lamellal signaling and an interdigitated undulating T cell:APC interface

A transient F-actin sheet drives lamellal signaling

With a substantial part of the T cell signaling system localized in the lamellal pattern it was imperative to understand its cell biological foundations and signaling function. In activating T cells, it is thought mostly based on work using planar APC substitutes (32, 41, 42, 62), that F-actin is generated at the interface edge pushing it outward during T cell spreading. Retrograde F-actin flow from the interface edge inwards is then used to move active receptor complexes toward the interface center that is mostly devoid of F-actin. However, during the peak of lamellal signaling as determined by super resolution microscopy of phalloidin-stained T cell:APC couples, we found that F-actin covered the entire interface at a depth up to 2 μm into the T cells with only slightly more F-actin at the interface periphery (Roybal et al., unpublished data). Live cell imaging with the F-actin sensor F-tractin (42) corroborated these data. Recent analyses of NK cell:target cell couples with superresolution microscopy reveal a similar F-actin distribution covering the entire interface with a modest enrichment at the interface edge (63, 64). Moreover, we found that lamellal signaling intermediates share their location with an F-actin network, at the population and single cell level,

that they move comparably as determined with fluorescence recovery after photobleaching experiments, and that they use defined molecular interactions for lamellar localization (K. T. Roybal and C. Wülfing, unpublished data). When we pharmacologically diminished interface F-actin accumulation through treatment with a low dose of Jasplakinolide, lamellar localization of actin and signaling intermediates declined in parallel. In addition, the activity of lamellar but not central signaling intermediates was selectively modulated (K. T. Roybal and C. Wülfing, unpublished data). Together these data establish that the lamellar pattern constitutes an F-actin network with embedded signaling complexes, where the lamellar localization of the signaling intermediates is required for their optimal activity. In contrast to the dynamic nature of lamellar patterning, central accumulation was sustained over minutes if not more, enriched molecules were close to immobile and early central accumulation was less actin-dependent (K. T. Roybal and C. Wülfing, unpublished data), suggesting a stable and highly cross-linked micrometer scale protein assembly, possibly similar to large protein complexes with distinct phase properties (65). These data suggest that the segregation of early T cell signaling into preferential central and lamellar localization is underpinned by two very different cell biological structures, a highly cross linked close to immobile large signaling complex at the interface center and numerous much smaller F-actin-linked dynamic complexes in the lamellum.

In the activation of T cells on planar surfaces the actin network can be separated into a lamellipodium at the very interface periphery that mostly consists of branched F-actin and a lamellum closer to the interface center characterized by actin-myosin II arcs (66). It is unclear whether such a clear structural separation also applies to T cell:APC couples. Consistent with T cell activation on planar surfaces, the distribution of Myosin II is slightly more central than that of the entire F-actin pool (KTR and CW, unpublished). However, myosin light chain kinase as a key myosin II activator and Myosin 1C are mostly peripheral and lamellar, respectively (KTR and CW, unpublished, (45)). We therefore use the term lamellar accumulation to reflect the pronounced extension of the T cell plasma membrane toward the APC that accompanies it but don't suggest that this structure is necessarily equivalent to the lamellum as defined in T cell activation on planar surfaces.

T cells must detect low doses of antigen (67-69) with only modest distinction from self (70, 71). Stable central signaling even in response to weak stimuli, as suggested by effective central PKC θ accumulation at limiting peptide concentrations (30), may provide a robust hub for sensitive core signal propagation, consistent with the highly cross linked close to immobile nature of central signaling. We suggest that the lamellum with smaller more dynamic complexes rich in signaling intermediates involved in signal modulation may be better suited to discriminate accidental or short-term receptor triggering from stronger antigen-specific sequential engagement over minutes (72-74) thus supporting specificity. In this scenario, consistent serial engagement of the TCR together with costimulatory receptors would drive the transient actin dynamics (75) that are required for the formation of lamellar signaling complexes. Being enriched in both actin regulators and signaling intermediates, these complexes could then provide positive feedback to amplify T cell signaling at its peak (75, 76). This positive feedback between actin dynamics and signaling would also be well suited to mediate kinetic proofreading in T cells (77).

Lamellar actin dynamics drive an undulating T cell:APC interface

Given the ability of F-actin to shape plasma membrane topology and given the importance of plasma membrane topology in shaping receptor:ligand engagement, the membrane topology of the T cell:APC interface is of great interest. Such topology can only be resolved by electron microscopy. Using endothelial cell cultures induced to express MHCII with IFN- γ and T cell activation with superantigen, T cells can extend projections into the endothelial cells, termed invadosome/podosome-like protrusions, whose formation coincides with the rapid elevation of the intracellular T cell calcium concentration and that serve as signaling foci with tight T cell:endothelial cell membrane contact at the tip of the invadosomes (78). Electron microscopy showed comparable membrane interdigitation in T cell:B cell and T cell:dendritic cell couples (78). At later time points, more than 15 min after initial T cell:APC contact, T cell:dendritic cell interfaces were tight and undulating with occasional deep protrusions into the APC (79). Undulating cellular interfaces were also seen in the interaction of cytotoxic T cells and NK cells with target cells (80-82). To relate T cell membrane topology to lamellar signaling, we studied 5C.C7 T cell:CH27 B cell couples by electron microscopy within seconds of cell couple formation. Numerous T cell lamella protruding into the APC resulted in a highly undulating cellular interface (K. T. Roybal and C. Wülfing, unpublished data)(Fig. 4). Based on measurements of the undulating interface outline relative to the planar interface diameter in single EM sections, the interdigitation of the cellular interface leads to a more than four-fold increase in the actual interface area over a flat disk representing its outer dimensions. Importantly, the same pharmacological interference with F-actin dynamics that reduces lamellar accumulation and signaling activity also impairs the membrane undulations (K. T. Roybal and C. Wülfing, unpublished data). In addition, with the dissolution of the lamellar pattern both interface actin amounts and the membrane undulations declined. Together these data strongly suggest that F-actin-driven membrane undulations are an integral part of the lamellar pattern.

The lamellar actin network and the undulating T cell:APC interface shape T cell signaling

The existence of a wide and deep signaling network that is driven by F-actin and associated with a highly undulating cellular interface should have profound consequences for T cell signaling. The more than four-fold increase in T cell:APC contact area resulting from the early membrane undulations should enhance ligand scanning. The dramatically increased membrane surface to cytoplasm volume ratio in T cell membrane extensions could alter signal progression as established in neurons (21, 83). A deep lamellar actin network may transmit cell surface signals via mechanotransduction along rigid actin filament assemblies to distant cellular compartments such as the nucleus at millisecond timescales (84). The deep T cell plasma membrane undulations are also highly deformed membrane structures that could affect actin polymerization (85) and molecular interactions at the membrane as Bin/Amphiphysin/Rvs (BAR) proteins maintain curved membrane structures and provide a link to various actin regulators (86). Intriguingly, the F-BAR-domain containing protein Cdc42-interacting protein 4 (CIP4) is critical for NK cell polarization in the interaction with target cells (87). The early T cell lamellum likely constrains actin-mediated transport. As the T cell/APC membrane interdigitation

breaks the T cell surface into numerous individual membrane extensions protruding toward the APC with narrow and deep invaginations between them, retrograde actin flow during early T cell activation should be directed towards the base of T cell invaginations, i.e. perpendicular to the interface plane (K. T. Roybal and C. Wülfing, unpublished data). Invaginations should also obstruct large-scale F-actin assemblies and cortical receptor movement towards the interface center. Thus early signaling clusters become trapped across the lamellum in a range of small sizes. Once the lamellar pattern has mostly dissolved, a flatter interface topology should be more conducive to lateral receptor/ligand transport that can build and/or sustain larger protein clusters (2, 88). While experimental corroboration for these suggestions is still missing, in controlling surface area, plasma membrane to cytoplasm volume ratio, membrane curvature and the direction of F-actin motion the lamellar actin network has to profoundly shape the cellular context of early T cell signaling in T cell:APC couples. Similar actin sheets with embedded signaling complexes may play a comparably important role whenever dynamic cell contacts drive cell fate decisions, such as in development (89), stem differentiation (90), and cancer metastasis (91). Many of the signaling intermediates enriched in the lamellar pattern in T cells are critical regulators of these processes (92, 93).

In contrast to the actin-driven membrane undulations that shape early T cell activation in T cell:APC couples, the interface between a T cell and a planar APC substitute is flat by design. The spatiotemporal organization of T cell signaling should thus differ substantially depending on whether a T cell is activated with an APC or a planar substitute thereof. Indeed, many differences exist. For example, central receptor clustering is almost instantaneous in contact with APCs (Fig. 3A) whereas it takes minutes to develop in contact with support lipid bilayers (6). The interface center is an area of dynamically regulated signaling activity in T cell:APC couples, it is thought to be virtually signaling dead in T cells activated on bilayers (40). In the activation of T cells by planar APC substitutes the entire early T cell signaling machinery from the TCR via ZAP-70 to SLP-76 first accumulates in microclusters at the periphery of the T cell/bilayer interface and then moves centripetally (3, 11). In T cell/APC clusters TCR accumulation starts at the interface center after transit through the distal pole, active LAT accumulates preferentially at the interface center, but SLP-76 transits from immediate central accumulation into the lamellar pattern. As a possible contribution to understanding these differences, molecular mechanisms have been identified where actin polymerization is dependent on membrane curvature. For example, enhancement of actin polymerization by phosphatidylinositol 3 phosphate through binding to the sorting nexin 9 in an *in vitro* actin polymerization assay occurred only on curved lipid surfaces of a diameter of 400 nm or less but not on planar surfaces (85). While the comparison between T cell organization driven by planar surfaces versus B cell APCs is by no means comprehensive, it already strongly supports the notion that in the interpretation of data obtained with APCs or planar APC substitutes it will be of great importance to consider the divergent biophysical constraints of both systems.

Actin-driven organization of signaling as a rheostat of T cell activation

Actin dynamics are a principal regulator of cellular organization. The discovery of the lamellar network provides a new critical means for actin to regulate T cell signaling

activity. This is now part of the more general notion that actin dynamics regulates the efficiency of T cell effector function, e.g. in cytokine secretion, through control of the spatiotemporal organization of signaling. Large scale correlative evidence supports this hypothesis: Signaling intermediates that are known to regulate the efficiency of T cell activation often also control interface actin accumulation and the spatiotemporal organization of T cell signaling including lamellar patterning and membrane topology. The general involvement of actin dynamics in the regulation of the efficiency of T cell signaling is certain, as T cells deficient in a variety of key components controlling actin turnover, such as WAVE2, WASP, HS1, Myosin II, Cofilin, or Coronin, all display suboptimal signaling (94-103). Moreover, many signaling intermediates that control the efficiency of T cell signaling, such as CD28, Vav1, or Itk, also enhance actin amounts at the cellular interface (33, 45, 104-106). Intriguingly, Itk-deficient T cells or T cells activated in the absence of CD28 engagement are also less capable of organizing their signaling systems into distinct spatiotemporal patterns (30, 45). This prominently and consistently includes a lack of lamellar accumulation ((30), e.g. SLP-76 Fig. S1S and T in (45)). Moreover, the T cell/APC interfaces early during T cell activation upon Itk-deficiency or costimulation blockade are less undulating (Fig. 4), similar to treatment with a low dose of Jasplakinolide. Thus upon Itk-deficiency or costimulation blockade the regulation of the efficiency of T cell activation in cytokine secretion occurred in parallel with the control of interface actin amounts and spatiotemporal organization including lamellar patterning and membrane topology. This relational network supports the notion that actin-dependent lamellar signaling organization is an important regulator of the efficiency of T cell activation.

Nevertheless, other contributions of actin dynamics are certainly conceivable. In contrast to immediate central accumulation within the seconds to minutes of cell coupling sustained central clustering in T cell signaling is dependent on intact actin dynamics (33, 107). Comparing different T cell/APC combinations and different T cell activation conditions the accumulation of signaling intermediates at the interface center was consistently related the efficient phosphorylation of key proximal signaling intermediates (30). Interestingly, the composition of the central signaling cluster and the role of some of its components seem to vary with the strength of T cell stimulation. While PKC θ was a consistent part of central clustering supporting a role in core signaling (30), the association of the TCR with the interface center was highly dependent on cellular activation conditions suggesting a limited function in signal integration with costimulatory receptors in a subset of T cells (108). Actin dynamics are also linked to receptor internalization: CD2AP binds to capping protein and cortactin and controls receptor internalization (109). Dynamin as a critical regulator of many forms of endocytosis controls actin dynamics and T cell signaling efficiency (110). Together these data start to identify specific cell biological processes used by actin dynamics in the regulation of T cell effector function through the organization of signaling at the system scale, the lamellar signaling network, sustained central clustering, and possibly control of receptor trafficking. We thus start to understand how actin-driven signaling organization can function as a critical rheostat of T cell function.

Functional roles of the spatiotemporal organization of T cell signaling

An investigation of the spatiotemporal organization of signaling allows the efficient discovery of the function of signaling intermediates and cell biological structures by placing them into the context of information processing inside live cells. Testing such spatiotemporally constrained hypotheses is a great challenge. Two avenues to corroborate the importance of spatiotemporal distributions are the generation of large-scale relational networks, such as the ones in support of a function of lamellar and sustained central signaling in the regulation of the efficiency of T cell activation, and direct manipulation of spatiotemporal features.

Such causal support for specific roles of spatiotemporal organization is still scant. This is largely because methods to manipulate the localization of parts of the T cell signaling system are just being developed and are generally more complex than methods to manipulate the absence or presence of a protein irrespective of its location. Optical methods hold great promise, as light can easily be directed toward a desired subcellular volume of a cell. Even though not yet applied in T cells, chromophore-assisted light inactivation (CALI) can in principle be used to ablate a protein at a specific time and location. The protein of interest is fused to a chromophore that upon irradiation generates sufficiently large amounts of reactive oxygen species, in particular singlet oxygen, to destroy the fusion partner at the time and location of irradiation (111). A genetically encoded chromophore, KillerRed, is available (112). Complicating the application of CALI, it is most efficient when the chromophore-tagged protein is used in the absence of the endogenous one. Alternatively, signaling processes can be activated at a given time and location by uncaging of a critical signaling activator with light. This has for example been used to control the time and location of TCR engagement through a caged photo-sensitive version of the agonist peptide in the determination of the rapid kinetics of T cell signaling following initial TCR engagement (113) and in the identification of roles of different PKC family members in T cell polarization (114). Here the need to synthesize a caged version of a critical signaling activator may be limiting. Protein interactions can in principle be controlled using optogenetic methods. Light responsive proteins (e.g. Phy and PIF(115) or Cryptochrome(116)) that dimerize and disassociate in response to irradiation with distinct wavelengths of light can be fused to two signaling proteins allowing for control of their interaction. Imaging systems have been built such that the ON and OFF wavelengths of light can be rapidly positioned in live cells at sub-micrometer resolution and light intensities can be varied to regulate timing, amplitude, and duration of signaling at millisecond timescales (115). As both potential interaction partners need to be expressed as tagged variants, a substantial amount of cellular engineering is involved.

In addition to optical methods, the location of a protein can be altered by fusion to functionally inert domains with a strong localization preference. For example, to test whether the absence of active Cdc42 at the center of the T cell/APC interface in Itk-deficient T cells was related to diminished interface actin accumulation, active Cdc42 was pulled back to the interface center in Itk-deficient T cells through fusion with the Tec PHTSH3 domain. Amounts of interface actin could thus be restored, even though likely with slowed actin turnover (45). Sometimes it can be exploited that in higher eukaryotes the same enzymatic reaction can be executed by a number of homologous enzyme family members that differ in their subcellular localization. For example, spatiotemporally distinct pools of PIP₂ generated through overexpression of different isoforms of phosphatidylinositol 5-kinase were used to show that localized

PIP₂ controlled TCR localization and IL-2 secretion but cell-wide amounts of PIP₂ controlled T cell rigidity (117). Control of the spatiotemporal features of the protein of interest is less precise with these genetic than with optical methods, however, they are easier to apply, in particular as they often work on the background of endogenous protein.

Outlook

More than a decade has passed since the initial discovery of uneven spatiotemporal distributions in T cell signaling by the Kupfer group (2) and a first glimpse of the spatiotemporal organization of T cell signaling at the system scale with underlying cell biological structures and causal corroboration of implied protein function emerges as discussed here. Nevertheless our understanding of how the T cell signaling system is organized in time and space and how such organization governs T cell function is still far from comprehensive. This is particularly unsatisfactory as the system scale spatiotemporal organization of T cell signaling by encoding information flow through control of interaction probabilities is a uniquely rich source of regulatory information that thus remains largely untapped. The current limited extent of spatiotemporal understanding of T cell signaling is largely the consequence of substantial technical challenges:

Spatiotemporal patterning needs to be addressed at the system scale, as the control of interaction probabilities in signaling is at the heart of its functional impact. When the number of system components analyzed grows, the number of interactions that can be investigated as a consequence and thus the signaling information that can be extracted increases much faster. Approaches for the efficient acquisition of spatiotemporal data on large numbers of T cell:APC couples are critical.

Most current imaging sensors visualize the distribution of the total amount of the protein under investigation. As the activation of a protein is generally driving its localization, these sensors regularly allow indirect access to active signaling processes. Nevertheless, sensors that can directly reveal the localization of different active forms of a signaling intermediate will be of great utility.

Images are incredibly information rich, with thousands of data points in each single image of a single T cell undergoing activation. The vast majority of this information is still not used. The development and application of efficient methods for the quantitative analysis of large image data sets is thus of great importance. System scale image quantification should play a major role in generating critical input data for the mathematical modeling of signaling. Given the complexity of signaling in the simultaneous interaction of dozens if not hundreds of signaling intermediates as constrained in time and space, mathematical modeling offers the only realistic chance for comprehensive understanding.

System scale imaging, by revealing information flow encoded in spatiotemporal organization, is a powerful means to generate hypotheses about how cellular function is regulated. However, the testing of spatiotemporally constrained hypotheses requires causal means to change the localization of components of signaling systems in conjunction with means to assess the organizational and functional consequences. The further development of methods for causal spatiotemporal manipulation of signaling is critical.

Current work mostly focuses on the first few minutes of T cell activation. While this is the time of most dramatic morphological changes, of the peak of

biochemical signaling activity, and of the translocation of transcription factors, T cell effector functions such as cytokine secretion can take much longer to develop as shown through experimental interruption of T cell signaling (118-120). While as little as 15 minutes of receptor engagement can be sufficient to reactivate a primed T cell, thus supporting current experimental time frames, hours of continuous signaling activity are required for the acquisition of effector function in naïve T cells, thus defining future challenges. Experiments to follow the spatiotemporal organization of T cell signaling until the onset of effector function, e.g. cytokine secretion, are thus of interest.

Once solutions to these challenges allow a comprehensive investigation of the spatiotemporal organization of T cell signaling as a function of different activation conditions or differentiation states, the emerging causally supported understanding of system scale signaling interactions promises unprecedented insight into mechanisms of the regulation of T cell function.

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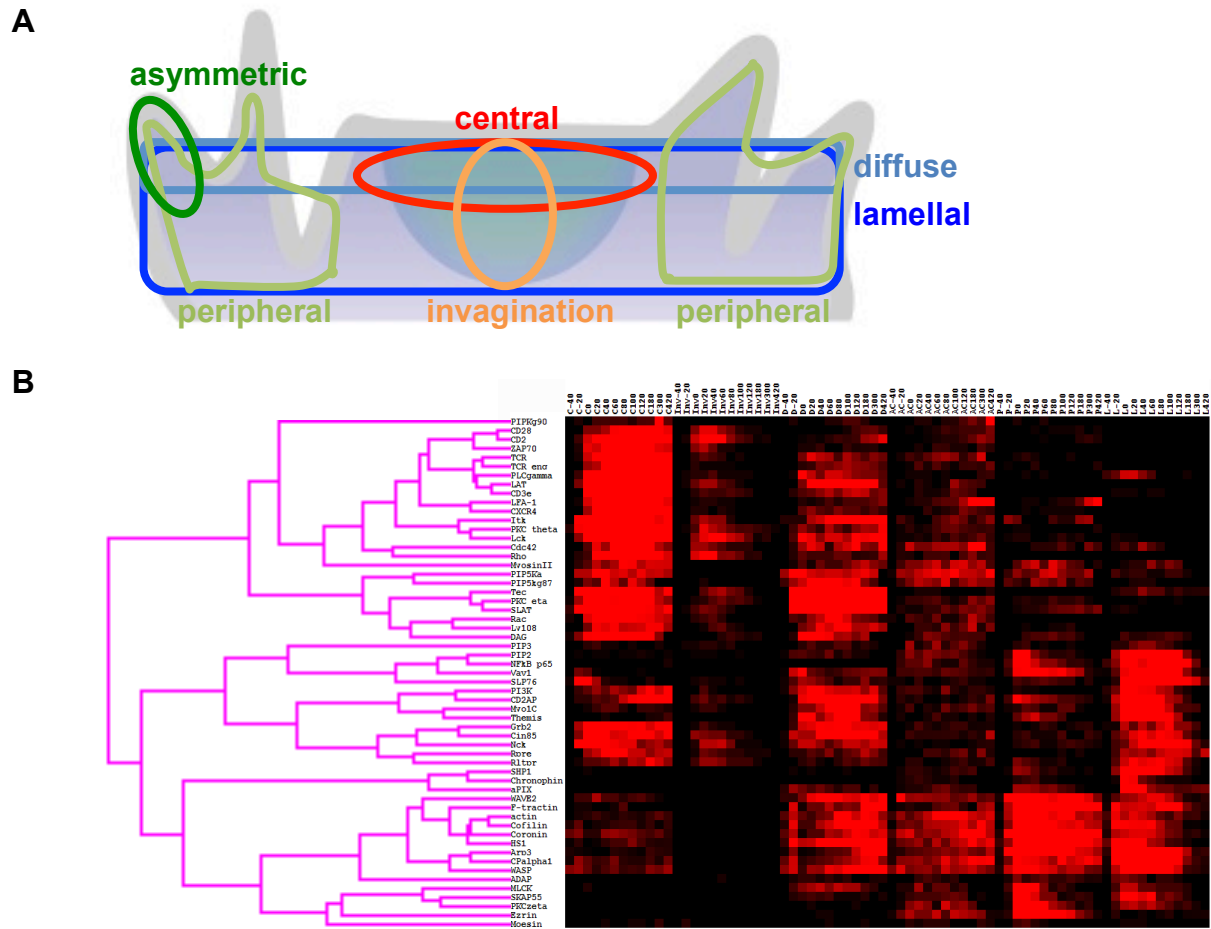


Figure 1 The spatiotemporal organization of T cell signaling is highly diverse. (A) The panel graphically represents the six categories used to classify spatiotemporal features of T cell signaling as defined in detail in (30, 45). The APC above the T cell is not shown. (B) 5C.C7 T cells expressing the indicated sensors were activated on peptide loaded CH27s (10 μ M MCC) and interface enrichment was scored in the six interface patterns [central (C), invagination (Inv), diffuse (D), asymmetric (AC), peripheral (P), and lamellum (L)]. The percentage occurrence of each pattern is given in shades of red from C-40 to L420. Representative imaging data and cluster analysis graphs are given in the previous publications (30, 45) and in Fig. 3. Spatiotemporal relations between sensors were determined by cluster analysis of the shown percentages of pattern occurrences and changes thereof between subsequent time points, as previously described (30), and are represented by the pink cluster tree. On average 59 cell couples were analyzed per condition (3318 total).

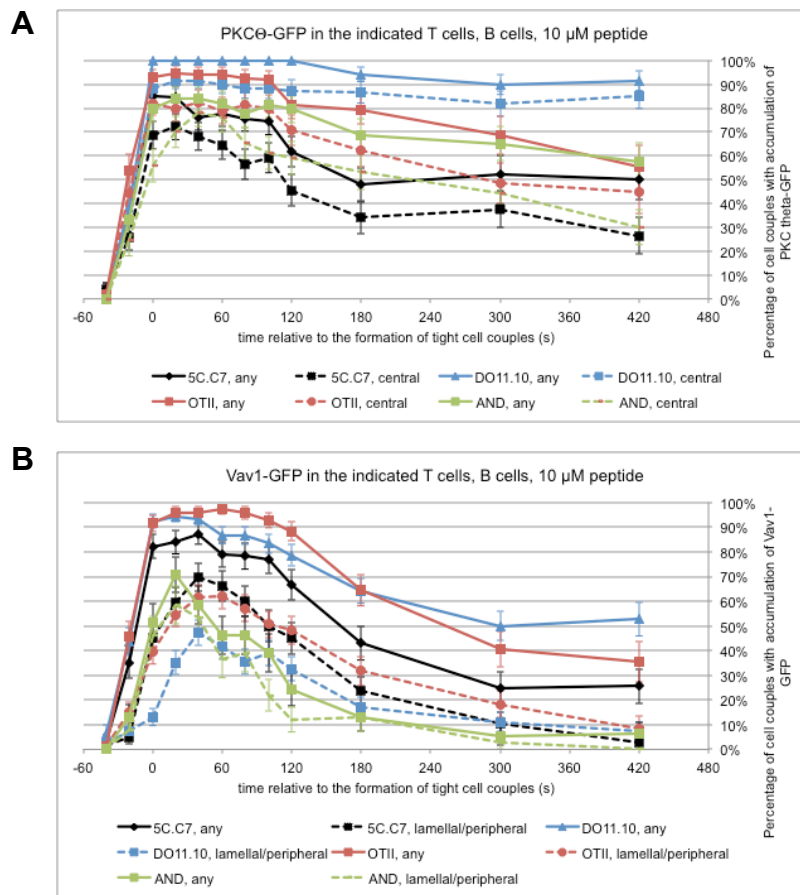


Figure 2 Spatiotemporal preferences are conserved across T cells with different TCRs. T cells from four different TCR transgenic mouse lines were activated as follows: primary in vitro primed 5C.C7 T cells, CH27 B cell lymphoma APCs, 10 μ M MCC 83-102 peptide; primary in vitro primed DO11.10 T cells, A20 B cell lymphoma APCs, 10 μ M Ova 324 – 340 peptide; primary in vitro primed OTII T cells, primary C57BL/6 B cell blasts, 10 μ M Ova 324 – 340 peptide; primary in vitro primed AND T cells, CH27 B cell lymphoma APCs, 10 μ M MCC 83-102 peptide. The accumulation of PKC θ -GFP in any interface pattern or in the central pattern (A) and the accumulation of Vav1-GFP in any interface pattern or in one of the two large actin-based patterns, lamellal and peripheral, (B) are given with SEM. On average 58 cell couples were analyzed per condition (472 total).

Figure 3

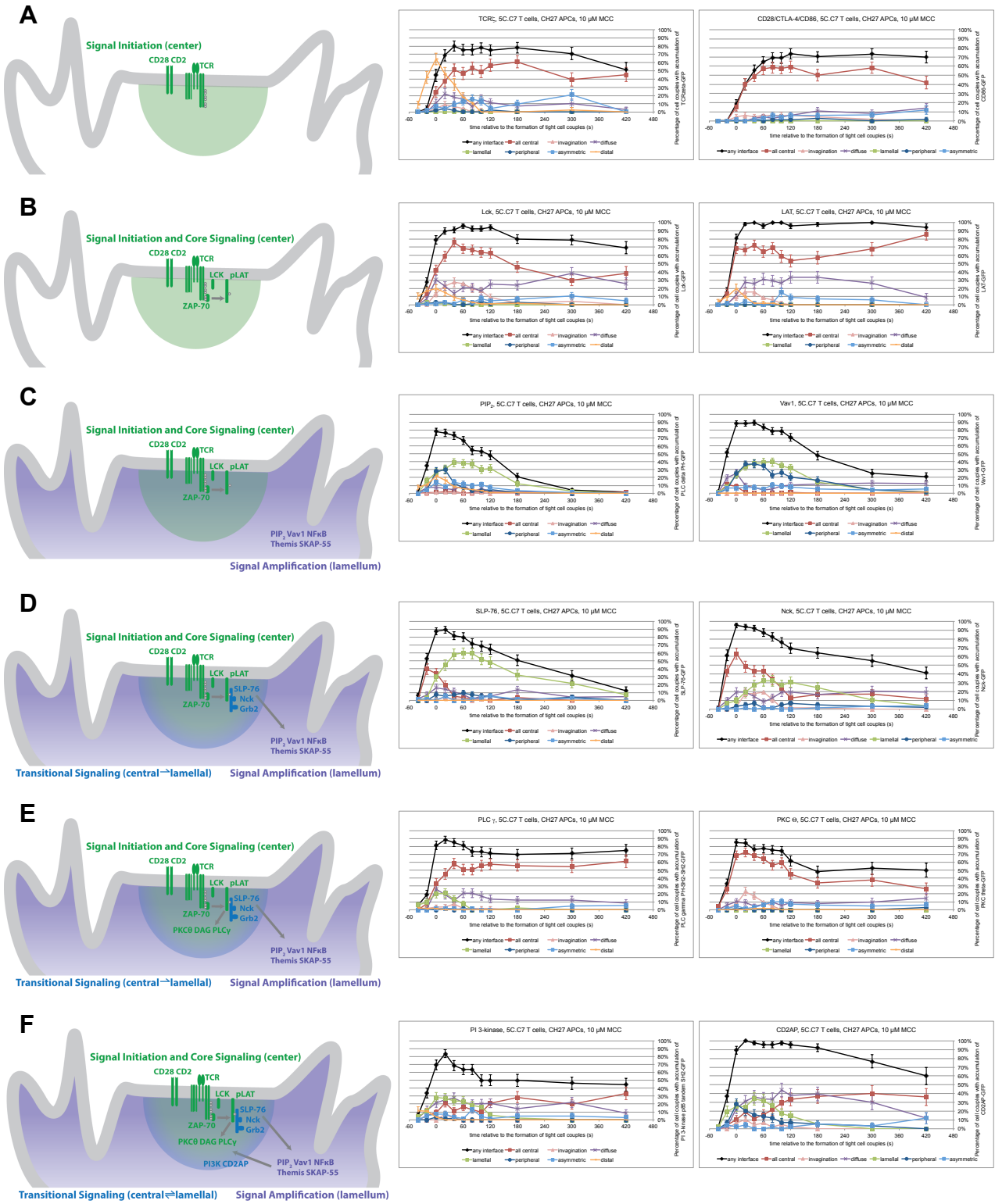


Figure 3 T cell signaling segregates in a dynamically connected fashion between the interface center and a transient actin-supported lamellum. The different stages in the spatiotemporal segregation of signaling transduction in T cells activated by B cell APCs are given schematically with representative pattern classification data. The APC-proximal part of the T cell is given with the APC on top not shown. Segregation is represented by signaling intermediates drawn at the center (central) or toward the side (lamellum) of the T cell. Dynamic transitions are indicated with grey arrows. The six stages of spatiotemporal signal progression are **(A)** signal initiation through receptor engagement, **(B)** receptor-proximal signaling, **(C)** signaling intermediates in signal amplification, **(D)** central to lamellal transition upon formation of the lamellal pattern, **(E)** core signaling, and **(F)** lamellal to central transition upon dissolution of the lamellal pattern. Each scheme is accompanied by representative patterning data for selected sensors. In these panels, 5C.C7 T cells were transduced to express GFP-based imaging sensors as indicated and activated with CH27 APCs and 10 μ M MCC agonist peptide. The graphs show the percentage of cell couples with standard errors that displayed sensor accumulation with the indicated patterns (30, 45)(Fig. 1A) relative to tight cell coupling.

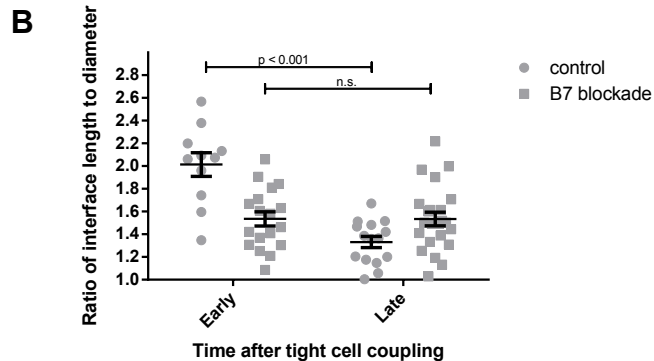
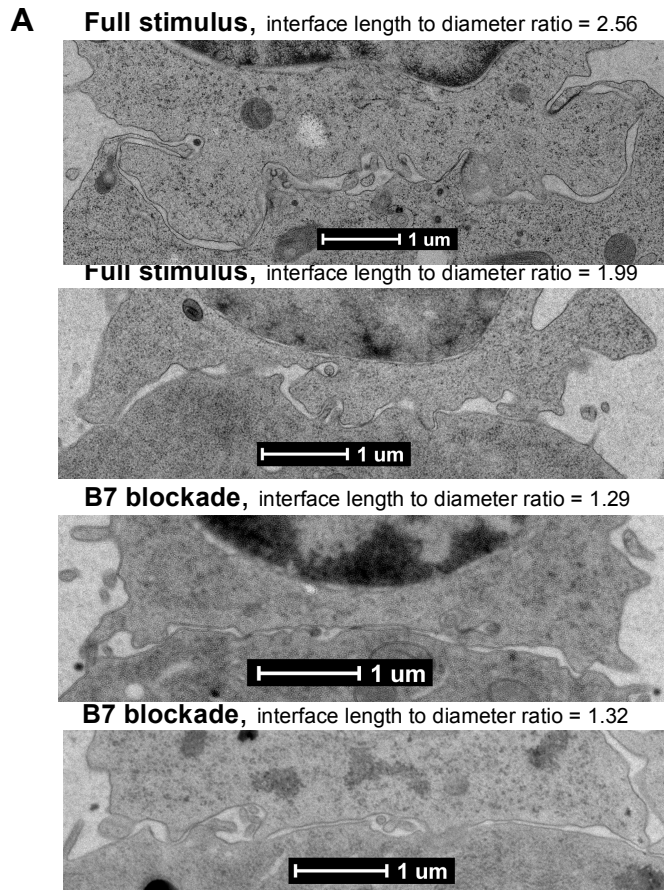


Figure 4 The T cell:APC interface becomes less undulating at the peak of lamellar patterning upon costimulation blockade or *Itk*-deficiency. **(A)** Representative electron micrographs of cellular interfaces of 5C.C7:CH27 APC cell couples (10 μ M MCC) at an early time point are given with the interface length to diameter ratios for wild type 5C.C7 T cells and upon costimulation blockade with 10 μ g/ml anti-CD80 and anti-CD86. T cells are on top, APCs on the bottom. **(B)** The corresponding interface length to diameter ratios for all cell couples analyzed are given for early and late time points. On average 34 cell couples were analyzed per condition (68 total).

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