New views of the immunological synapse: variations in assembly and function

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The interaction of T cells with antigen-presenting cells results in the formation of a contact face, termed the immunological synapse. The prototypical dynamics of this process are well established and involve cessation of crawling, a highly fluid 'immature' synapse phase during which signaling is initiated, and ultimately the formation of a 'mature' synapse characterized by centralized and peripheral supramolecular activating complexes. Ongoing research is directed towards defining how these supramolecular assemblies are formed and, more importantly, to what end. With regard to the former, progress has been made in defining the order in which various molecules are recruited to signaling centers in prototypical settings. With regard to the latter, however, the issue now appears more complex, as both developmental changes in T cells and variations in the environment appear to modulate features of mature synapse development. Although many details of the immunological synapse have been established, emerging evidence suggests a great variability in the ultimate form of these contacts and their effects on T-cell functions.

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Abbreviations

APC	antigen-presenting cell
CD2AP	CD2-associated protein
c-SMAC	centralized SMAC
CTL	cytotoxic T lymphocyte
CTLA-4	CTL-associated antigen 4
DC	dendritic cell
GFP	green fluorescent protein
IFN	interferon
IL	interleukin
LAT	linker for activation of T cells
p-SMAC	peripheral SMAC
SMAC	supramolecular activation cluster
TCR	T-cell receptor
WASP	Wiskott-Aldrich syndrome protein
ZAP-70	zeta-chain-associated protein 70

Introduction: the prototypical mature immunological synapse

Although the concept of an immunological synapse has been around for many years [1], it is only in the past five years that the forms of this interface have been appreciated. Monks, Kupfer and colleagues [2] first noted that the T-cell receptor (TCR) does not uniformly accumulate at the interface between a T cell and its antigen-presenting cell (APC), but is frequently found with a centralized distribution, which they named a centralized supramolecular activation cluster (c-SMAC). The term 'immunological synapse' was subsequently applied to the formation of a c-SMAC (TCR engaged by MHC molecules) surrounded by a peripheral SMAC (p-SMAC) of integrins [3]. More recently, with the realization that calcium signaling [4] and protein kinase signaling [5] precedes the segregation of the SMACs, this formation has been re-termed the 'mature immunological synapse' to differentiate it from the short-lived and highly fluid 'immature' phase that precedes it. A hallmark of the mature immunological synapse is its formation from the highly dynamic receptor microclusters observed in an immature synapse [3,4] as well as its relative stability — evidence of a c-SMAC can often be observed at least one hour after coupling has commenced [3,6•].

How to build a synapse

Scanning

T cells are exquisitely sensitive to low levels of peptide and rely on several mechanisms to boost their responsiveness. In defining the level of this sensitivity, a recent report by Davis and co-workers [7••] has demonstrated that as few as 10 agonist peptides are required for the formation of a c-SMAC, and as few as one peptide might be able to mediate a longer contact time between the T cell and the APC, inducing transient signal generation. The significance of this lies in the paucity of ligands required — previous estimates suggested that more than 100 contacts were necessary to generate activation of T cells [8]. Additional work by Davis and colleagues [9] might provide an explanation for this exquisite sensitivity — high concentrations of extremely low affinity ligands ('null' single-amino acid variants of the agonist peptide presented by the syngeneic MHC) are able to provide a boost to T-cell activation in response to low

concentrations of agonist. This is mediated by MHC interactions, as demonstrated by the finding that high-level accumulations of null-bearing MHC molecules are detected in the immunological synapse together with agonist-bearing complexes [7••,9]. Physiologically, this provides a mechanism for activating T cells against a low abundance peptide by relying on a profusion of secondary interactions with other, potentially self-derived, peptides.

Proximal kinase activation

Recent studies have shed light on the dynamics of signaling molecule distribution within the first minutes of T cell–APC contact formation — the immature synapse phase.

Previous biochemical evidence suggested that the initiation of signaling is regulated by the kinase lck, and much attention has been given to the dynamics of its activity. Total lck, as tracked with green fluorescent protein (GFP) fusion proteins, appears rapidly in the synapse, although additional pools are later recruited from endosomal stores [10]. The analysis of lck phosphorylation with specific antibodies reveals the complex regulation of its activity. In naïve T cells, the phosphorylation of lck and zeta-chain-associated protein 70 (ZAP-70) is detected before the formation of a mature synapse phenotype, and at early time points these kinases are mainly localized at the periphery of the synapse [5]. A further study from Shaw and colleagues [11] has shown that the activation of lck by peptide-pulsed APCs requires the stimulation of CD4 and CD28: CD4 is important for lck recruitment and CD28 is important for sustaining lck phosphorylation.

Further information on the regulation of lck localization and activation is demonstrated by independent experiments tracking the distribution of CD4 and the TCR. It is clear from kinetic and fluorescence resonance energy transfer (FRET) studies that CD4, similar to lck, is recruited to the TCR at early time-points [12]. In the mature synapse, however, it seems that the bulk of CD4 is relegated to the periphery [4] and lck colocalizes with it there [10]. This suggests that some lck might associate with CD4, even after the onset of signaling, and follow it out of the c-SMAC.

The dynamics of lck and ZAP-70 phosphorylation has also been analyzed by Kupfer and colleagues [13], who revealed a transient loss of tyrosine phosphorylation at the c-SMAC at early time-points. This phenomenon could, in part, be explained by the dynamics of CD45 (a phosphatase for lck) localization: CD45 initially redistributes to the c-SMAC concurrent with a transient dephosphorylation of TCR-associated substrates. However, lck is recruited to the c-SMAC early (within three minutes of contact in this study) and is then retained in the c-SMAC even at intermediate time-points (seven minutes), when CD45 is observed predominantly in a 'distal' SMAC. Finally, after 23 minutes of contact, lck loses its c-SMAC localization.

Signalosome assembly

In addition to lck localization, the assembly of complex multimolecular structures are observed early during synapse formation. Upon TCR triggering, using anti-CD3 immobilized on glass coverslips, Bunnell *et al.* [14••] demonstrated the recruitment of ZAP-70, linker for activation of T cells (LAT), Grb2, Gads and SH2-domain-containing leukocyte protein of 76kDa (SLP-76) to the sites of TCR clustering within 30 seconds of contact with the stimulatory surface. Although the association of ZAP-70 with these clusters was maintained for at least 20 minutes, the adaptor molecules LAT, Grb2 and Gads re-diffused within minutes, suggesting that they don't participate in later c-SMAC functions; this, however, remains to be established under circumstances in which c-SMACs actually form. Interestingly, SLP-76 moved into a cluster at the center of the contact area with the stimulatory coverslip even though the TCR ligands were immobilized on this surface [14••].

Although the recruitment of signaling molecules to the sites of TCR engagement is well established, the mechanisms by which these events regulate the redistribution of molecules that leads to the formation of a mature synapse have not yet been well characterized.

The role of the cytoskeleton and motor proteins in synapse assembly

It is now generally believed that, at least in part, active cytoskeletal-driven mechanisms are responsible for the synapse assembly that follows initial T-cell stimulation (see also Update). A further indication that this is an active process stems from a recent study in which the speed of TCR reorientation into the contact face was calculated from imaging data and found to be significantly higher than predicted by simple diffusion [15].

More specifically, actin cytoskeleton poisons, such as cytocalasin D, and general myosin motor inhibition disrupt the synapse formation process [4,16]. In addition, deficiency or mutations in several molecules involved in regulating the actin cytoskeleton, such as Vav, Cdc42 and Wiskott-Aldrich syndrome protein (WASP), cause defective synapse patterns [17]. More recently, by selectively targeting actin polymerization through the introduction of a truncated WASP protein in T cells, an inhibition of TCR–MHC accumulation at the c-SMAC was observed [18].

The importance of WASP in the formation of the synapse has been further demonstrated in Wiskott-Aldrich syndrome (WAS) patients. In T cells lacking WASP, impaired raft clustering at the synapse is detected

together with defects in calcium fluxes [19]. A possible linkage between WASP and the synapse machinery might be through the recently reported interaction with CD2, CD2-associated protein (CD2AP) and proline-serine-threonine phosphatase-interacting protein-1 (PSTPIP1). Following CD2 engagement these molecules form a complex that promotes actin polymerization and synapse formation [20].

The identity of the motor proteins involved in synapse formation is still elusive and, although it has been suggested that the class II myosins play a role in mediating receptor accumulation at the synapse, our recent findings suggest that this myosin subfamily is, in fact, dispensable for synapse formation (J Jacobelli and MF Krummel, unpublished); therefore, other myosin members are likely to be key players in this process.

The role of co-stimulation

Initial [16,21] and more recent [9,22] work has demonstrated a potential role for co-stimulation in synapse assembly; however, the mechanisms by which this is achieved remains unclear. Wulfing and colleagues [18] have recently argued that co-stimulation has a direct role in synapse formation on the basis of actin–GFP dynamics in the presence of CD28 and leukocyte function-associated antigen 1 (LFA-1) co-stimulation, particularly at lower antigen doses. By contrast, Bromley *et al.* [23] failed to detect any significant effect of CD28 ligation by B7-1 on MHC recruitment and synapse assembly in a lipid bilayer system. Whereas initial reports also suggested that co-stimulation recruited lipid raft components [21], this too is now in dispute [24]. Although a possible explanation of the different findings could derive from the different experimental systems used in these studies, another explanation may lie in the mechanism by which CD28 'co-stimulates' T cells — an issue that is still not completely understood.

Although CD28 stimulation might induce specific signaling cascades, this has never been unambiguously demonstrated. A wealth of evidence is emerging to support a role for CD28 as a signal integrator — a transmembrane adaptor protein that, by tethering multiple signaling molecules at the synapse, can promote downstream activation programs. Work by Acuto and colleagues [25] shows that, particularly at lower TCR stimulation levels, CD28 signaling increases calcium influx and phospholipase C γ1 (PLCγ1) activation in an Itk-dependent manner, suggesting that CD28 signals integrate via the TCR during proximal signal generation. Recent data showing that CD28 and TCR recruitment correlate with calcium influx also support the hypothesis that CD28 has an early role in T-cell activation, before the assembly of a mature synapse, by boosting calcium signaling and prolonging T cell–APC interaction (PG Andres and MF Krummel, unpublished). Taken together, these studies suggest that the effects of CD28 on synapse assembly might derive principally from its role in assembling Itk, phosphatidylinositol 3-kinase (PI3K) and PLCγ1 into a signaling unit on the CD28 intracellular tail.

In contrast to the distribution of CD28, the inhibitory B7 counter-receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) is enriched in lipid rafts [26] and redistributes to the synapse only during the late stages of T-cell stimulation [27]. Notably, the extent of this CTLA-4 recruitment to the synapse seems to require high levels of T-cell stimulation, possibly providing a mechanism for negative feedback to regulate strength of signals and T-cell activation [27]. Furthermore, the observation by Chikuma *et al.* [28] that CTLA-4, by interacting with CD3ζ, can limit TCR levels within the lipid raft compartment suggests a mechanism by which this molecule could affect TCR signaling.

Why build an immunological synapse?

Despite much work on the issue, we are left with an open question — what is the purpose of the mature immunological synapse? Below, we have sought to outline the potential answers to this problem and the recent evidence in support of them.

Prolong signaling

The concept that the mature immunological synapse promotes prolonged signal integration has received much attention. Kupfer and co-workers [2] first suggested that the c-SMAC was associated with full activation and, indeed, was necessary for that response. Studies using weak agonists have demonstrated that they do not permit stable receptor microcluster formation in the immature synapse, they do not recruit and cluster tyrosine-phosphorylated proteins [29], and they do not result in c-SMAC formation. More recent work by Huppa *et al.* [6•] supports a model in which prolonged signaling and c-SMACs are interconnected. Using the pleckstrin homology (PH) domain of Akt fused to GFP as a reporter to measure phosphatidylinositol (3,4,5)-trisphosphate (PIP3) levels in the contact face suggested that PI3K activity is sustained for at least 10 hours, well after the majority of the TCRs have been internalized. However, the continued engagement of the TCR during these periods was demonstrated by blocking the specific peptide–MHC complex at various times post-contact and showing that PIP3 levels rapidly decayed. A mechanism by which continual TCR ligation might contribute to signaling while undergoing internalization is suggested by the observation that TCR stimulation leads to increased surface transport of TCRs, effectively supplying the cell with more receptors to facilitate signaling [30].

Thus, it would appear that prolonged TCR engagements accompany the late stages of a mature synapse, and it is likely that these would extend the duration of active

transcription at key loci such as IL-2. Nonetheless, the absolute requirement for a c-SMAC-like assembly at these late stages could not be addressed in these experimental settings, and it remains possible that the active TCRs in this system represent a very small number that signal outside the c-SMAC.

Coalescence of signaling pathways

As discussed previously, the c-SMAC is a place where multiple receptors (e.g. TCR–CD3, CD2, CD28) all co-localize. One benefit of such close localization is that the signals generated may integrate in ways that individual microclusters might not reinforce. Such a 'signalosome' is supported in the work of Bunnell *et al.* ([14••], discussed above) in which the successive recruitment of molecules to sites within the cells is observed. The dynamics of such an assembly could have profound effects on the recruitment of specific linkers, such as the members of the membrane-associated guanylate kinase (MAGUK) family, and thus upon the functional outcome of the complex (see [31] for review).

Directed granule release and cytokine secretion

One logical function of a polarized interface is to direct the secretion of lytic granules and cytokines during the interaction with target cells or APCs respectively. Directed killing (by $CD8^+$ cells) or help (by $CD4^+$ cells) would be necessary to maximize the efficiency and minimize unwanted bystander interactions.

Jenkins and colleagues [32] demonstrated that *in vivo* IL-2 stores in $CD4^+$ T cells are oriented towards the adjacent APC, confirming the initial *in vitro* observation of cytokine polarization by Kupfer and colleagues [33]. More recently, Griffiths and colleagues [34] have shown that a specialized synapse is formed between cytotoxic T lymphocytes (CTLs) and their target cells. In these synapses, lytic granules polarize at the site of the microtubule-organizing center (MTOC) and are secreted through a specialized domain, devoid of TCRs, encircled by the integrin p-SMAC ring. Because the interaction between the CTL and the target cell during cytotoxicity is relatively brief and is sensitive to very low doses of antigen [35•], it is possible that these synapses never proceed to a completely mature phase, allowing the rapid and repeated killing of multiple targets. In addition, these synapses appear to permit the transfer of membrane markers from target cells to the CTLs during the killing process [34]. This phenomenon, which is also observed in other studies, has been proposed as a mechanism of regulating the CTL response by inducing a 'fratricidal' killing of CTLs that have acquired enough antigenic peptide–MHC complexes from target cells.

Provide a contact face for weaker ligands

Recent evidence has shown that both CD28 and CD40 typically do not accumulate at sites where their ligands are present unless the TCR simultaneously binds to the same surface (J Boisvert and MF Krummel, unpublished; [23]). A clear implication is that the synapse, and specifically the integrin upregulation and spatial segregation of receptor ligands, corrals these lower-affinity interactions and permits prolonged repetitive binding. Taken together, this would allow TCR recognition, by virtue of its ability to form a c-SMAC, to dictate the signaling of independent ligand–receptor pairs — in essence 'licensing' these signals to be delivered.

Downregulate signaling

The c-SMAC is thought to be a logical place from which TCRs internalize, and recent evidence has arisen to support this contention. A cytoplasmic linker protein CD2AP/$p130^{Cas}$ ligand with multiple Src homology 3 (SH3) domains (CMS) was found to associate with the transmembrane receptor CD2, which segregates to the c-SMAC. CD2AP also inducibly associates with the ubiquitin E3 ligase c-Cbl, suggesting that it might recruit the ubiquitination machinery to activated receptor complexes [36]. The phenotype of the CD2AP knockout in T cells was recently analyzed by Shaw and co-workers [37•] and was found to have a defect in c-SMAC formation and a concomitant defect in TCR downregulation following TCR engagement. Notably, total tyrosine phosphorylation was both spatially localized to the peripheral zone of the synapses in these cells and temporally prolonged relative to control cells, suggesting that CD2AP is necessary for the regulation of signal duration. T cells from these animals proliferated more robustly than control cells [37•]. It remains to be determined whether c-SMAC formation, prolonged signaling and the absence of internalization are in a linear pathway or are independent events that rely on CD2AP function. For example, CD2AP has multiple SH3 domains and also associates with cortactin [38], a cytoskeletal connection that may aid the coalescence of receptors, independently of its role in recruiting c-Cbl.

Variations on the theme

Although the fundamentals of synapse development continue to emerge, another theme has arisen, suggesting that synapse formation might not be a fundamental process for all relevant T-cell contacts. This has resulted from two lines of investigation.

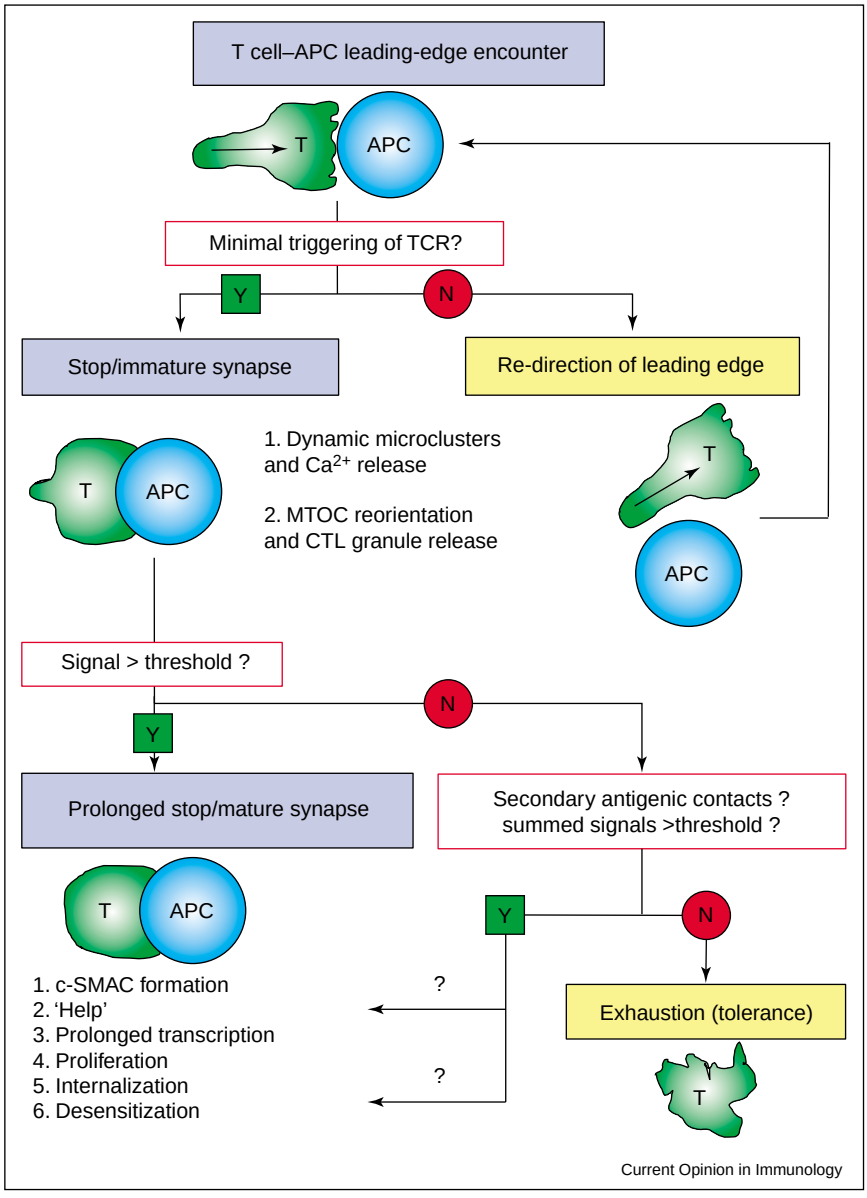
Naïve T cells *in vivo*

Experiments in which T cells were placed together with purified dendritic cells (DCs) in an *in vitro* collagen matrix first suggested that stable dynamics are not requisite for T-cell recognition [39]. Such experiments demonstrated that naïve TCR-transgenic T cells formed only transient contacts with the DCs with short bursts of calcium signaling, and yet were able to proliferate *in vitro* in response to these repeated stimuli.

Analysis of naïve T cells reacting in a live or explanted lymph node *in vivo* has further challenged the paradigm of a stable mature immunological synapse. Miller *et al.* [40••,41] demonstrated that naïve $CD4^+$ T cells actively scan through the lymph node and, in the presence of administered antigen, slow their speed of crawling to a rate consistent with interaction with lymphoid APCs. However, these interactions appear to be transient in the first days and only later result in a 'swarming' behavior suggestive of a semi-stable interaction. In similar experiments in which DCs were also labeled, Robey and co-workers [42•] found that interactions of $CD8^+$ T cells with antigen-bearing DCs last in the order of hours or longer.

More recently, von Andrian and co-workers [43•] separately labeled both $CD4^+$ T cells and antigen-presenting

Figure 1



A sequential decision-making tree for the functional outcome of T cell–APC interactions. T cells may have multiple chances to generate an above-threshold signal. At each node, the strength of signal acts as a checkpoint to dictate the degree to which downstream signaling and synapse assembly will occur. Included in this model is the ability of T cells to sum sequential sub-threshold signals in order to ultimately generate a strong transcriptional response. At present it is not clear whether sequential interactions can ultimately generate stable synapses or whether complete activation can always be achieved in the absence of such structures. Insufficient sub-threshold encounters may ultimately result in T-cell exhaustion and tolerance. N, no; Y, yes.

DCs, and demonstrated that naïve T cells tend to initially favor very short interactions with individual APCs, similar to observations by Friedl and co-workers of T cells in collagen matrices [39]. However, as the systemic response progresses over the first 24 hours, T cells begin to favor longer interactions. Furthermore, on day two post-immunization, these appear to revert back to transient engagements. The reason for this shift is not clear — it is possible that either T cells, their presenting cells or perhaps an element of the milieu undergo changes in the intervening time period. To this latter possibility, Dustin and co-workers [44] provided evidence to suggest that certain soluble chemokines might prevent T cells from stopping in response to TCR stimulation, a result that may be consistent with conditions in which stopping is minimized *in vivo*. Taken together, these results emphasize that the dynamics of T-cell encounters are not consistently the same from T cell to T cell. This also calls into question some results in which synapse dynamics were studied in naïve T cells cultured *in vitro* — such cells typically round-up when removed from the lymph node, perhaps due to the absence of chemokine signals, and must be centrifuged to promote stable contacts with APCs [5].

The peptide-specific generation of a stop signal that, in itself, typically does not lead to complete synapse formation in the work of Davis and co-workers [7••], suggests that the transient T cell–APC interactions observed *in vivo* by the laboratories of Cahalan [40••,41] and von Andrian [43•] are reflective of relatively insensitive T cells or APCs bearing low levels of antigens. Part of the answer to how T cells might then effectively surpass the threshold for mature synapse formation with such transient or weak interactions may lie in their ability to 'sum' sequential signals (Figure 1). A paradigm for this exists for mast cells that can sum, spatially or temporally, agonist signals and ultimately release their histamine granules when a super-threshold signal is generated [45]. Faroudi *et al*. [46] have suggested that a similar mechanism might be at work in T cells — the blockade of T-cell activation during stable encounters using PP2, a reversible src-kinase inhibitor, resulted in dissolution of the nascent synapse. This could re-assemble when the inhibitor was removed. However, multiple engagements followed by dissociations did ultimately result in IFN-γ production by $CD4^+$ T cells. Although this system is far from physiological, and the proliferative outcome of such a system is unknown, the result clearly suggests that T cells have the ability to temporally sum inputs generated by sequential bursts of signaling.

Synapses in the thymus

Results of the analysis of the immunological synapse in thymocytes are equally revealing. In two separate studies, thymocytes undergoing negative selection on supported lipid bilayers or in thymic reaggregate cultures did not form a single centralized TCR cluster. Rather, they formed a collection of smaller clusters localized within or to the periphery of the central zone, whereas similar stimulation of T-cell blasts results in prototypical c-SMACs [47,48] Together with tyrosine phosphorylation patterns in the thymocyte synapse that are also reminiscent of an immature synapse, this suggests that these cells do not progress to a mature synapse, but are maintained in an immature phase.

Notably, no receptor aggregation was observed in either system using positively selecting stimuli. In this case, 2-photon imaging proved revealing — thymocytes responded to a positively selecting stimulus in explanted thymus lobes, similar to naïve T cells in the first hours of the response to agonist ligands in explanted lymph nodes. Thymocytes underwent rapid movement punctuated by long-lived cellular associations displaying stable cell–cell contacts and shorter, highly dynamic contacts, only in the presence of a positively selecting APC.

Unified fate maps for T cells

The results discussed above all suggest that the synapse is an integration mechanism, the details of which are just being worked out. In Figure 1, we outline the various bifurcations of the decision-making process. It should be emphasized that, although the events outlined here have been observed *in vitro* and *in vivo*, the actual details of how and why the decisions are made largely remain to be discovered. However, it is interesting to speculate that sub-threshold stimulation, manifested as multiple unstable interactions, might ultimately 'burn out' the signaling machinery, resulting in exhaustive tolerance.

Conclusions

The next generation of experiments is likely to further clarify the variations and functions of the individual stages of synapse formation. It will be particularly interesting to observe differences from established themes and determine the role of each molecular formation in shaping the immune response. So, it now appears likely that the dynamics of T-cell interactions with antigen-bearing cells may ultimately be shown to vary considerably according to a number of parameters. These might include the developmental status of the T cell, the nature of the antigen and APC and the milieu in which the encounter takes place. However, the inclusion of proximal components into signaling assemblies has been initially mapped. The challenge now is to determine when additional components are recruited, how they are recruited and what function they serve in generating diverse types of supramolecular complexes and downstream responses. It will be particularly interesting to observe the differences from established themes and determine what role individual molecules and molecular assemblies play in shaping the immune response.

Update

Recent work from Faure *et al*. [49] has shown a direct linkage between Rac activation and the function of ezrin-radixin-moesin (ERM) proteins. In this system, inactivation of the latter results in relaxation of the actin cytoskeleton. During synapse development this may be required to provide a flexible membrane with which to efficiently conjugate to the APC, and for patterning of ERM-associated molecules such as CD43.

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Now in press

The work referred to in the text as (J Jacobelli and MF Krummel, unpublished) is now in press [50]. The work referred to in the text as (PG Andres and MF Krummel, unpublished) is now in press [51].

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