

T-CELL-ANTIGEN RECOGNITION AND THE IMMUNOLOGICAL SYNAPSE

Johannes B. Huppa and Mark M. Davis

Much excitement of the past five years in the area of T-cell-antigen recognition has centred around the immunological synapse — a complex cellular structure that forms at the interface of a T cell and a cell that expresses the appropriate peptide–MHC complexes. Thanks to new imaging technologies, we are now beginning to understand the role of cell-surface molecules and some of their attendant signalling modules in the context of cell-to-cell communication. Progress has been so rapid that T-cell-antigen recognition might be the first system in which the molecular basis of cell–cell recognition is understood.

T cells have a crucial role in orchestrating the body's adaptive immune response, and much effort has been put into defining the types of T cell that exist and their functions. Many of the cell-surface and cytoplasmic molecules that are involved in the various forms of T-cell recognition, activation and effector function have been identified and characterized. Although progress in defining the molecular steps that are involved in T-cell signalling and activation has been steady, it has remained less clear how the cell-surface molecules on T cells interact with each other and their ligands to mediate different stages of recognition. Neither classical biochemistry nor classical cell-biological approaches have been able to explain adequately how a T cell can scan a large number of other cells, find one with the appropriate antigen and carry out a complex activation process that can take many hours. Instead, T-cell biologists have taken advantage of new imaging technologies (BOX 1) to piece together a new approach to the problem of cell–cell recognition, one in which a biochemist's interest in the molecular underpinnings of a phenomenon can be integrated with a cell biologist's understanding of what is happening where at the whole cell level.

Much curiosity has centred around the discovery of what is now known as the immunological synapse — the area of cell contact. Schematically illustrated in FIG. 1, this synapse shows the highly complex and

ordered distribution of molecules that was first observed between T helper (T_H) cells and B cells.

What is a synapse?

The word 'synapse' is derived from the Greek words meaning 'connection' or 'junction' between two similar entities (Oxford English Dictionary). It was first used to describe the junction between two chromosomes in the late 1800s and shortly afterwards was used for neuronal connections. The term immune synapse was first chosen by M. Norcross¹ to describe T-cell–antigen-presenting cell (APC) interactions and also by W. Paul and colleagues². Here, we define it as any stable, flattened interface between a lymphocyte or natural killer (NK) cell and a cell that they are in the process of recognizing. Conceptually, this term puts the activation of these cells in the context of a highly organized and dynamic structure that can act as a platform for a bidirectional and cell-specific flow of information, and that might offer additional layers of modulation to a cell's response.

Here, we highlight the most recent findings in this area, and a summary of the model systems used to generate these data is given in BOX 2. Furthermore, we attempt to draw a more holistic sketch of antigen recognition by outlining examples that illustrate the interconnectivity between ligand binding, signal generation and signal integration. We conclude by discussing the possible significance of the immunological synapse for T-cell responses.

The Howard Hughes Medical Institute and The Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305, USA. Correspondence to M. M. D. e-mail: mdavis@pmgm2.stanford.edu
doi:10.1038/nri1245

Box 1 | Approaches to study the immunological synapse

Wide-field epi-illumination fluorescence microscopy

A common method of illumination in fluorescence microscopy, in which the illuminator is placed on the same side of the specimen as the objective lens, and the objective carries out a dual role as both a condenser and an objective.

Pros: fastest data acquisition, large field of view, wide range of excitation wavelengths

Cons: haziness of images due to out-of-focus light, image de-convolution required to achieve good image quality

Applications: live imaging of fast events such as the activation of effector T cells

Confocal laser scanning microscopy (CLSM)

A mode of light microscopy in which a focused laser beam scans the specimen in a raster and the emitted fluorescent light or reflected light signal, sensed by a photomultiplier tube, is displayed in pixels on a computer monitor. A variable pinhole aperture, located in a plane confocal with the specimen, rejects out-of-focus light and allows for optical sectioning.

Pros: excellent image quality

Cons: slower image acquisition, often limited choice of excitation wavelengths

Applications: ideal for slow or non-moving (for example, fixed) specimens

Spinning (Nipkow) disk confocal microscopy

Uses a Nipkow (spinning) disk that is impregnated with holes as a means to transfer an image onto a CHARGE COUPLED DEVICE (CCD). The scanning disk contains multiple, symmetrically placed spirals of pinhole apertures through which light is passed and split into multiple 'minibeams'. When spun, the light scans the sample in a raster pattern. Sample emission is detected to form an image on the CCD.

Pros: good image quality, fast image acquisition (video rate for smaller images), reduced photobleaching

Cons: reduced flexibility in the choice of excitation wavelengths

Applications: live-cell imaging

Two-photon and multi-photon laser scanning microscopy

Uses an infrared laser beam, the energy density of which allows the doubling or tripling of frequency at the point of beam focus in the specimen, for fluorochrome excitation. Molecules that simultaneously absorb two (or three) photons at 900 nm emit the same fluorescence as if they were excited by a single higher energy photon of 450 nm (or 300 nm). The method allows deep tissue penetration (~350 microns). Photobleaching and phototoxicity is minimized because fluorescence emission is contained within a single focal plane.

Pros: highest tissue penetration (up to 350 microns compared with 80 microns for conventional CLSM), least phototoxicity/photobleaching for most fluorophores (excitation wavelength >400 nm)

Cons: expensive, slow image acquisition

Applications: best for *in vivo* microscopy of tissues (such as lymph nodes and fetal thymic organ cultures)

Fluorescence lifetime imaging microscopy (FLIM)

FLIM takes advantage of the decrease in the lifetime of donor fluorescence when FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) occurs between a pair of fluorophores. Because only the lifetime of the donor fluorophore is measured, spectral bleed-through does not add to the background.

Pros: best way of FRET detection, especially when using communicating fluorophores with overlapping emission spectra (for example, cyan fluorescent protein, CFP and yellow fluorescent protein, YFP)

Cons: not yet commercially available

CHARGE COUPLED DEVICE (CCD). A slab of silicon semiconductor that is divided into an array of pixels that function as photodiodes and a light-sensitive photodetector.

The immunological synapse takes shape. The first evidence for cross-talk between receptor-mediated signalling, cytoskeletal reorganization and directed transport of cell-surface receptors came from studies that used soluble antibodies to crosslink T-cell receptors (TCRs) and other cell-surface molecules for lymphocyte stimulation. Such treatment resulted in a phenomenon described as 'capping', in

which cell-surface receptors, filamentous actin and lipids such as gangliosides congregate towards one end of the cell³. Immunofluorescence studies on fixed T-cell-APC conjugates by Kupfer *et al.*^{4,5} indicated a marked polarization of the T cell towards the B cell, in particular the movement of the microtubule organizing centre (MTOC) from the far side of the T cell to a location underneath the synapse. Of note, this cytoskeletal rearrangement is not just a consequence of T-cell stimulation, but seems to be required for sustained TCR-mediated signalling, as the actin-depolymerizing drug cytochalasin D interferes with calcium influx — a prominent feature of T-cell activation after TCR ligation⁶. Together, these studies provided the first evidence for both the complexity of the immune synapse as well as its significance for T-cell activation, although the latter remains controversial (see later).

Through 'optical sectioning' of a T-cell-APC conjugate, Kupfer and co-workers⁷ subsequently visualized these cells for the first time in three dimensions. Importantly, it was shown that key molecules such as the TCR and the adhesion integrin leukocyte function-associated antigen 1 (LFA1) were not only capped at the interface, but also were organized in distinct areas within the interface (FIG. 1a). These areas were termed supra-molecular activation complexes (SMACs) (FIG. 1b). The central region of the SMAC (cSMAC) is enriched in TCRs and one of its downstream signalling effectors, protein kinase C- θ (PKC- θ)⁷. Cell adhesion seems to predominate in a peripheral ring that surrounds the cSMAC — the pSMAC — which shows a high surface density of LFA1, as well as the cytoskeletal linker talin⁷. Large and bulky molecules such as CD43 and CD45 were localized in a region distal to the synapse outside the pSMAC — known as dSMAC.

What are the mechanisms that enable the site-specific transport of molecules to create such orderly assemblies, and what is the significance of macroscopic segregation for antigen recognition? One logical way to address these questions is to study the dynamics of synapse formation. This was greatly facilitated by new advances in live-cell-imaging technologies. Early efforts using video microscopy together with calcium imaging provided the first insight into the morphological changes that occur after the activation of T_H cells^{8,9}. Another advance came with the addition of molecular tags to key cell-surface molecules that enabled the observation of the rapid clustering of intercellular adhesion molecule 1 (ICAM1) linked to green fluorescent protein (GFP) in the context of T_H-cell activation¹⁰. In a technical tour de force, Dustin and colleagues^{11,12} exposed T_H cells to labelled MHC molecules and other molecules in artificial model membranes, and used confocal microscopy to monitor the formation of a synapse. These latter studies also illustrate the power of being able to follow a single T cell from the initiation of activation to stable synapse formation. This enables better correlations between a given stimulus and its consequences to be established.

FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET). An imaging technique to analyse intermolecular distances based on the transfer of energy from a donor molecule to an acceptor molecule without the emission of a photon.

Ligand threshold for synapse formation

T cells are highly sensitive to antigen, with estimates ranging from 1–400 specific peptide–MHC complexes on a presenting cell required to activate fully at least some T cells in a population^{13–15}. There are major uncertainties with these studies however: they rely on average values of peptide loading on a given MHC molecule and there is no way of measuring just how much surface area is ‘surveyed’ by a given T cell and how far it gets in the activation process. To illustrate the limitations of this bulk-cell population approach (as opposed to a single-cell approach), suppose you had detected full activation in a few T cells at an average of 100 peptides per APC. Not only will you have outliers in the APCs that might have 300 peptides per cell (and which might be responsible for most of the activation events when peptide is limiting), but it also matters a great deal whether the T cell surveys 1% of the APC surface or 50%.

An important recent advance has been the development of a new approach that provides more precise information about T-cell sensitivity and the nature of T-cell responsiveness to different numbers of ligands¹⁶. Here, the peptide extends out of the peptide-binding groove and is labelled on an amino-terminal biotin with a streptavidin–phycoerythrin conjugate. Phycoerythrin is a large (240 kDa) multimeric protein that contains ~34 chromophores¹⁷. Because of its brightness, it is possible to visualize single phycoerythrin molecules with a

standard cooled charge coupled device (CCD) camera¹⁸. In this way, it is possible to count the exact number of ligands that a T cell encounters on another cell, and then monitor the consequences of that interaction with respect to the increase of intracellular calcium concentration and the behaviour of different GFP-labelled proteins. In this way, it was shown that at least some CD4⁺ T-cell blasts can respond to even a single ligand, by stopping and fluxing calcium weakly (FIG. 2a). Two ligands in the interface produce a more sustained rise in calcium and ten or more ligands produce a maximal response and promote the formation of a stable synapse. Parallel experiments using two different cytotoxic T-cell models give a similar dose-response curve (M. Purbhoo *et al.*, unpublished observations). So, in at least four T-cell model systems, T cells can detect just one ligand, but all require about ten to increase and maintain calcium levels fully (FIG. 2b). Increased calcium levels are pivotal to T-cell activation, as this induces the nuclear localization of nuclear factor of activated T cells (NFAT) — a transcription factor that is responsible for many of the gene-expression changes associated with activated T_H cells¹⁹.

Importantly, 25–30 peptide–MHC complexes were required at the interface to induce T cells to stop and flux calcium when CD4 was blocked (FIG. 2b). These data indicate that CD4 is intimately involved in detecting antigens at low surface densities, which prompted

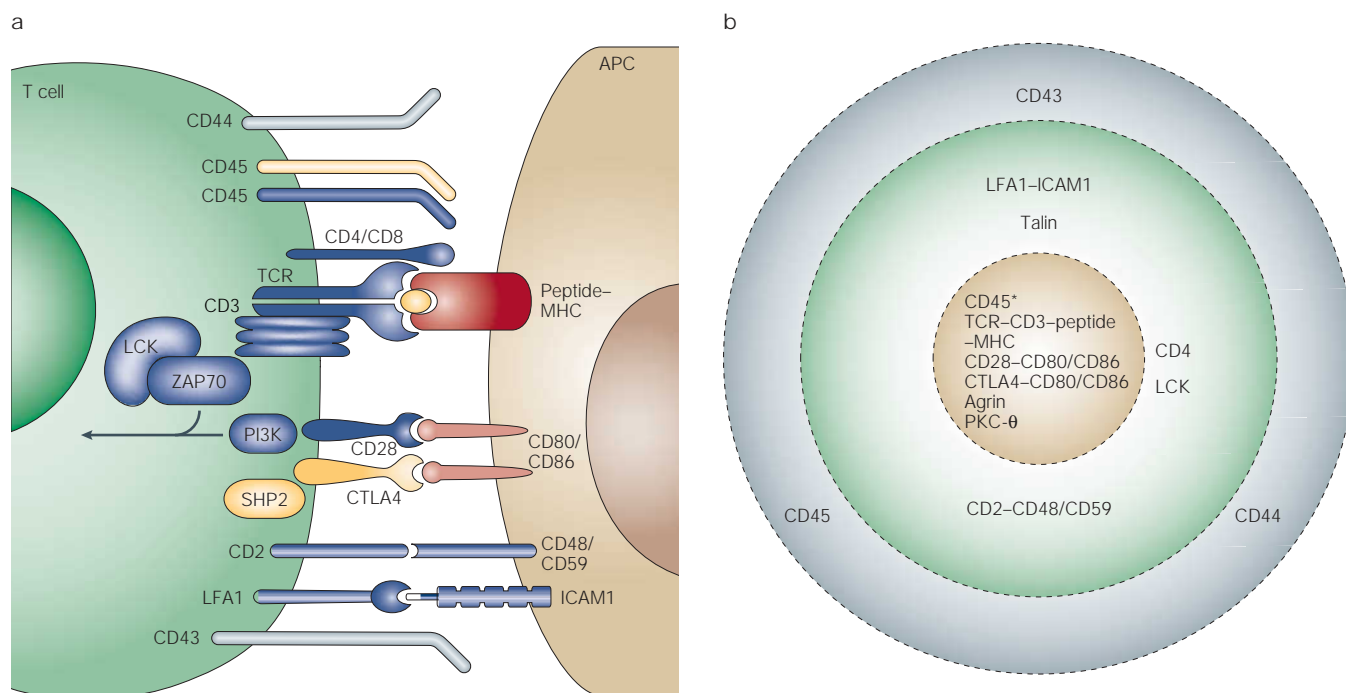


Figure 1 | Overview of a mature T-cell synapse. a | A profile view showing a selection of the key ligand pairs and signalling molecules that are involved in T-cell recognition. The stimulatory peptide–MHC molecule is shown in red, activating/co-stimulatory molecules are blue, inhibitory molecules are yellow and molecules that are not contributing to signalling are grey. The arrow indicates converging signals that lead to T-cell activation. **b** | The face on view of the synapse with the characteristic ‘bull’s-eye’ zone pattern, including the central region of the supra-molecular activation complex (cSMAC) (yellow), the peripheral ring surrounding the cSMAC (pSMAC, green) and the region distal to the synapse outside the pSMAC (dSMAC, grey) as well as the molecules/ligand pairs that are found enriched within. APC, antigen-presenting cell; CTLA4, cytotoxic T lymphocyte antigen 4; ICAM1, intercellular adhesion molecule 1; LFA1, leukocyte function-associated antigen 1; PI3K, phosphatidylinositol 3-kinase; SHP2, SRC homology 2-domain-containing protein tyrosine phosphatase 2; TCR, T-cell receptor; ZAP70, ζ-chain-associated protein 70. *CD45 enters the cSMAC at later stages.

Box 2 | T-cell–APC model systems: pros and cons

Jurkat T cells and Raji B cells plus superantigen

Easy handling and somatic mutant lines are available for many genes, however Jurkat cells are deficient for phosphatase and tensin homologue (PTEN) and so maintain high levels of 3' phosphatidylinositides, even, when not activated. In addition, these cells lack several adhesion and accessory molecules that are typical of normal cells, rendering them a 'non-physiological' model. Activation has to occur in the presence of superantigen as the nominal antigen is unknown.

T-cell hybridomas

Antigens are well defined, but cells often show non-physiological behaviour, possibly as a result of cell fusion.

Stable T-cell clones and T-cell lines

Antigens are well defined and most clones/lines are susceptible to either gene transfection or retroviral expression. However, there is often a marked degree of physiological variability (sensitivity, kinetics of activation, expression level of cell-surface receptors, for example).

Primary (*in vitro* stimulated) T cells

A fair approximation to the physiological scenario. With the appearance of retroviral gene transduction, ectopic expression of GFP-fusion proteins is now feasible.

***In vivo* imaging**

The best approximation of T-cell physiology. Spatial resolution of molecular distribution is reduced due to lower signal intensity.

Planar lipid bilayers and coverslip-immobilized surface proteins/antibodies

Having a structure such as an immunological synapse reduced from three to two dimensions enables high-image resolution and the use of interference-reflection microscopy (IRM) and TOTAL INTERNAL REFLECTION MICROSCOPY (TIRFM). The ability to define these lipid bilayers allows for a 'purify and reconstitute' approach, but only on the antigen-presenting cell side of the interface.

TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY (TIRFM). TIRFM is used to observe molecule fluorescence that is restricted to surfaces and interfaces and, therefore, is increasingly used to investigate the interaction of molecules with surfaces.

LIPID RAFTS

Specialized membrane domains in the plasma membrane that are enriched in a subset of glycolipids, cholesterol and certain proteins containing either a particular transmembrane domain or which are post-translationally modified with saturated acyl chains. Their exact properties (size, existence of sub species) and function in living cells is debated.

FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP). The diffusion coefficient and the mobile fraction of the detected species can be determined by FRAP where a small region is irreversibly bleached once with a short, intense laser pulse and the subsequent kinetics of the fluorescence recovery in the same bleached volume is recorded.

Irvine *et al.*¹⁶ to suggest a 'pseudodimer' model, in which CD4 crosslinks two TCRs, one which binds to an agonist peptide–MHC complex and a second that binds weakly to an endogenous peptide–MHC complex, a large number of which (~20%) seem to be compatible with TCR binding and are recruited into the synapse. Alternatively, it might be that ligation of only one ligand, together with the delivery of the tyrosine kinase LCK to the site by CD4 or CD8 is sufficient to initiate a signalling cascade. Most studies on the initiation of T-cell activation by soluble peptide–MHC class II complexes, however, show a requirement for dimers or higher order multimers^{20,21}. Interestingly, monomeric stimulatory MHC class I ligands seem to stimulate CD8⁺ cytotoxic T lymphocytes (CTLs) quite efficiently, however only when these cells were made adherent in an integrin-dependent manner (to fibronectin or CD18/CD11-specific-antibody-coated cover slips)^{22,23} and not when left in suspension²⁴.

Formation of a mature synapse

Ligand recognition causes the T cell to stop migrating and to form an increasingly stable cell contact with the corresponding APC. As schematically shown in FIG. 3, this involves the reorientation of the MTOC (as well as its associated vesicles), and the recruitment of receptors and signalling molecules to the nascent immunological synapse.

Molecular recruitment: role of co-stimulation. Early thinking about how the TCR and other molecules accumulated at the synapse centred around the idea of random diffusion of membrane molecules and trapping by ligand ligation²⁵.

Recent work, however, has indicated that this is an active (that is, energy dependent) process in which cell-surface molecules from all over the T cell are transported to the synapse through cytoskeletal linkage and molecular motors. This was first shown through the use of beads bound to T-cell membranes²⁶. Both TCR signals and CD28- or LFA1-mediated co-stimulation was required for bead movement. A similar transport phenomenon involving LIPID RAFTS was also observed²⁷. More recent work could directly link this type of transport to the accumulation of MHC molecules and synapse formation²⁸. So, one unexpected role of the 'second signal' in T-cell activation, is the transport of membranes, and probably a subset of their associated proteins, to the immunological synapse, and this is probably an important factor in its formation.

Lessons from artificial APCs. To account for mechanisms that might explain molecular segregation within the synapse, imaging efforts needed to be extended to visualizing the dynamics of specific proteins of known function. As mentioned earlier, one important approach to this issue has come from the use of artificial lipid bilayers that contain fluorescent-labelled T-cell ligands to activate T_H cells. T cells form ordered synapses with such membranes, and proliferate in an antigen-dependent manner as long as co-stimulation is provided for example, through ICAM1 (REF. 12). ICAM1 — the ligand of the T-cell integrin LFA1 — begins to cluster in the centre of the synapse and moves rapidly to a peripheral location. By contrast, peptide–MHC complexes are first found in a peripheral ring, but accumulate within minutes in the centre of the synapse¹². Of note, the density of peptide–MHC complexes in these central structures was found to be proportional to the half-life of monomeric TCR binding in solution, which usually correlates with the signal strength of the interaction. FLUORESCENCE RECOVERY AFTER PHOTOBLEACHING (FRAP) experiments showed lateral immobility of these peptide–MHC complexes, indicating a continuous engagement by TCRs. A simple model for the correlation between TCR half-life and ligand density in the synapse would be that it is a function of direct binding and efficient (or inefficient) dragging into the cSMAC, but recent work has shown that a strong agonist can trigger the surprisingly robust recruitment of poor TCR ligands to the site of T-cell contact, as long as stimulatory ligands are also present²⁸. This is not consistent with the model described earlier. We have, therefore, proposed a reostat model, in which a small quantity of the best ligands available determines the speed of TCR transport²⁸.

Does molecular size matter? Signalling events are tied to active cytoskeleton-dependent recruitment mechanisms (and vice versa), but do they alone account for the

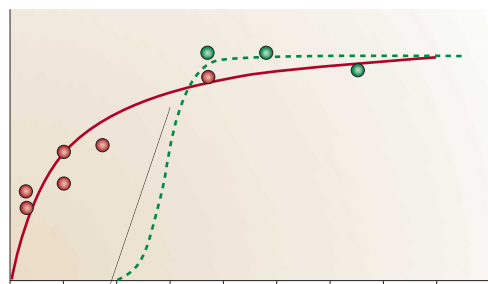
Function could be generated by co-segregation of similarly sized molecules, which then act together. Indeed, increasing the length of the co-stimulatory molecule **CD48**, which acts together with peptide–MHC–TCR binding by interacting with the accessory molecule **CD2**, has an inhibitory effect on T-cell activation — a finding that is consistent with a ‘size-matters’ hypothesis³¹.

Mathematical models have been developed to ‘predict in hindsight’ the peripheral location of the large ICAM1–LFA1 ligand pair (42 nm) and the central location of the much smaller peptide–MHC–TCR pair (15 nm) on the basis of large-scale self-organization^{32,33}. A case for such a similar-size-driven scenario had been proposed for the cross-talk between the peptide–MHC–TCR pair and the larger CD45 polypeptide. CD45 phosphatase activity maintains T-cell-antigen sensitivity by rendering the TCR-proximal kinase LCK active, yet CD45 has also been suggested to simultaneously have an inhibitory effect on T-cell activation through direct dephosphorylation of activated CD3 subunits³⁰. CD45 should be segregated from activated TCR–CD3 complexes for reasons of size — a state that would also facilitate T-cell activation. In a study using artificial bilayers for T-cell stimulation, CD45 was indeed excluded at an early stage from the central TCR zone and was found to be enriched in the most peripheral dSMAC^{34,35}, however, it clustered with the TCR in the pSMAC at later stages³⁴. Immunolocalization studies on fixed cell conjugates showed a transient recruitment of CD45 into the pSMAC, and fluorescence resonance energy transfer (FRET) indicated a molecular association between CD45 and TCR in the cSMAC³⁶. In summary, for CD45 and the TCR, a causal relation between molecular size, synapse location and function could not be established.

Similarly, CD43 — a heavily glycosylated and bulky molecule of unknown function — was found to be excluded from the synapse³⁷. But CD43 interacts through members of the **EZRIN–RADIXIN–MOESIN (ERM) FAMILY** with the actin cytoskeleton. Deletion of the ERM region resulted in an even synaptic distribution of CD43 with no obvious effect on the segregation of smaller receptor–ligand pairs nor on T-cell activation^{38–41}.

Therefore, although the hypothesis that molecular size affects cross-talk within the immunological synapse and helps to localize particular molecules is an attractive one, there is not as yet any compelling evidence that it has such a role.

Agrin and MGAT5. *Agrin* and *MGAT5* (mannosyl α 1,6-glycoprotein acetyl β 1,6-N-glucosaminyltransferase 5) were recently identified as genes that influence the degree of molecular segregation. The secreted glycoprotein agrin — originally isolated from the neuromuscular junction in which it helps to control the clustering of the acetylcholine receptor — has also been discovered in T cells in which it influences the clustering of various cell-surface receptors⁴². Whereas a heavily glycosylated form of agrin was found to be evenly distributed over the plasma membrane of resting T cells, a less glycosylated form that is predominant



macroscopic segregation of synaptic polypeptides? One parameter that might contribute to this behaviour is the size of the extracellular domains of various membrane proteins and that of their ligands. Proponents of this hypothesis argue that smaller, but stable, binding pairs might exclude larger proteins from their immediate vicinity^{29,30}. Vice versa, smaller membrane proteins might be prevented from interacting with their cognate receptors when surrounded by larger molecules, especially if space is limited in the cleft of a synapse^{29,30}. Adjacent ligand pairs that were different in size would impose a curvature on the membranes in which they are embedded, which is thermodynamically less favourable.

EZRIN–RADIXIN–MOESIN (ERM) FAMILY

The ERM family consists of three closely related proteins ezrin, radixin and moesin, which provide a regulated linkage between the cortical actin cytoskeleton and certain transmembrane proteins in the plasma membrane. There is increasing evidence that ERM family proteins participate in signal transduction.

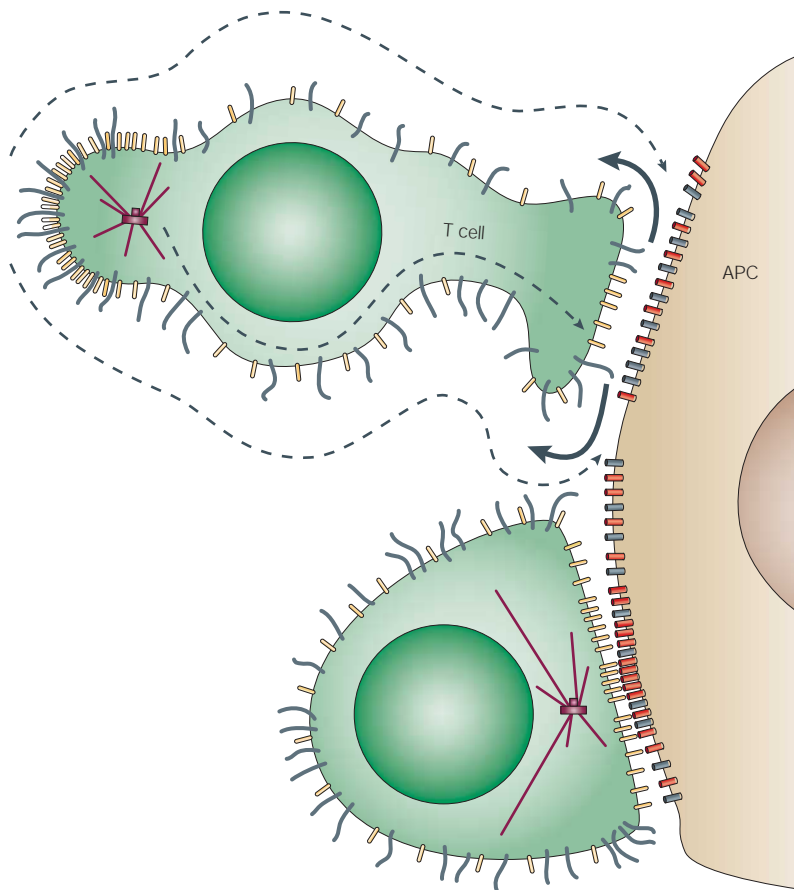


Figure 3 | Morphological and cytoskeletal changes in the initial engagement and formation of a stable T-helper-cell synapse. After initial engagement of the T-cell receptor (TCR) with its cognate peptide–MHC complex a T cell stops migrating and the microtubule organizing centre (MTOC) is reoriented from the uropod to beneath the immunological synapse. TCR molecules (yellow) are recruited into the synapse and other cell-surface molecules (for example, CD43) are excluded. Stimulatory (red) and non-stimulatory (grey) peptide–MHC complexes are present in the synapse as indicated.

on activated T cells co-localizes with clustered TCRs in the cSMAC. The addition of agrin purified from activated T cells in picomolar concentrations induced the polarization of TCRs, CD28 and membrane lipid rafts on resting T cells. The precise role of agrin in T-cell activation and how it mediates its effects on receptor clustering and raft aggregation remain to be identified. However, future studies involving agrin-deficient T cells will be required to determine the importance of agrin for T-cell function *in vivo*.

An important regulatory role for *N*-glycan synthesis in T-cell activation has recently been shown by Dennis and colleagues⁴³. Deficiency in the Golgi-resident glucosaminyltransferase MGAT5 — an enzyme involved in the *N*-glycosylation pathway — lowers T-cell activation thresholds by directly enhancing TCR clustering. MGAT5 function is required for the biosynthesis of tetra-antennary glycan structures that end with *N*-acetylactosamine — a disaccharide that binds to galectins with an affinity that is comparable to that of the peptide–MHC–TCR interaction (0.1 mM). When added exogenously, galectin-3 associates with subunits of the TCR–CD3 complex on the cell surface

and also with several other cell-surface proteins. A galectin–glycoprotein network has been proposed, which might restrict the mobility of cell-surface receptors including the TCR, therefore, raising the threshold for TCR signalling. Interestingly, transcription of the *MGAT5* gene was increased 48 hours after T-cell stimulation, indicating that MGAT5 enzyme activity is limiting in resting T cells and its activation-induced upregulation could function to negatively influence T-cell sensitivity to antigen at later stages.

Signalling within the immunological synapse

As T-cell activation changes the makeup of the synapse, communication between the conjugated cells is, in many cases, likely to be dynamic and mutual, rather than static and unidirectional. As it is intimately linked to the formation, maintenance and termination of the immunological synapse, T-cell–antigen recognition must be iterative and subjected to circumstantial parameters, which include the quality and quantity of the antigen, the type and developmental stage of the T cell and APC, the microenvironment in which these cells meet and perhaps even previous ‘experience’ of both the T cell and APC. Translating these parameters into molecular and cellular behaviour has long been a daunting task, which is only now becoming accessible by using new imaging approaches. These include visualizing activated signalling proteins in fixed conjugates, real-time recording of the recruitment behaviour of signalling molecules at sites of receptor ligation as well as the localized production of secondary messengers.

TCR and CD4: not always acting together. A three dimensional ‘optical sectioning’ approach together with live-cell imaging, calcium monitoring and GFP–gene fusions showed a surprising divergence in the behaviour of the TCR–CD3 complex and CD4 (REF. 44). Whereas both TCR-associated CD3 ζ –GFP and CD4–GFP accumulated rapidly after contact with an APC in dense patches in the contact area, only CD3 ζ –GFP accumulated in a cSMAC at later times. By contrast, CD4–GFP redistributed to the periphery in the course of several minutes. In a study carried out using a T-cell–hybridoma model system, which measured the physical interaction between TCR and CD4 by FRET, CD4 was diffusely enriched over the entire contact area⁴⁵. Therefore, these studies indicate a particular role for CD4 in signal initiation.

Recruitment dynamics of signalling molecules. Live-cell studies on the dynamics of TCR-proximal signalling were pioneered in readily transfectable tumour lines. Activation-induced recruitment of ζ -chain-associated protein 70 (ZAP70)–GFP from the cytoplasm to the plasma membrane was first shown in HeLa cells^{46,47}. Real-time ligation of TCR has been recently related to the buildup of signalling assemblies in Jurkat T cells stimulated with plate-bound antibody⁴⁸. Using FRAP, the authors showed that recruitment of ZAP70 to the TCR–CD3 complex is by no means static, but involves rapid cycles of binding and release.

Retroviral and lentiviral gene-transfer methods now make it feasible to image signalling proteins fused to GFP in primary T cells. Studies on both GFP-labelled⁴⁹ and antibody-stained fixed T cells show that LCK first localizes to the cSMAC and then redistributes to the periphery³⁶. In T-cell blasts (but not in naive T cells), a large proportion of LCK was found in internal vesicles associated with the MTOC, which translocated to the interface within the first ten minutes after cell contact⁴⁹. Whether this pool of LCK contributes to T-cell signalling remains to be determined.

Early peak in TCR-proximal signalling. The kinase activity of LCK and ZAP70 requires phosphorylation of specific tyrosine residues (Tyr394 of LCK, and Tyr492 and Tyr318 of ZAP70), and phospho-specific antibodies have been generated to track the location of active kinases in fixed conjugates by immunofluorescence.

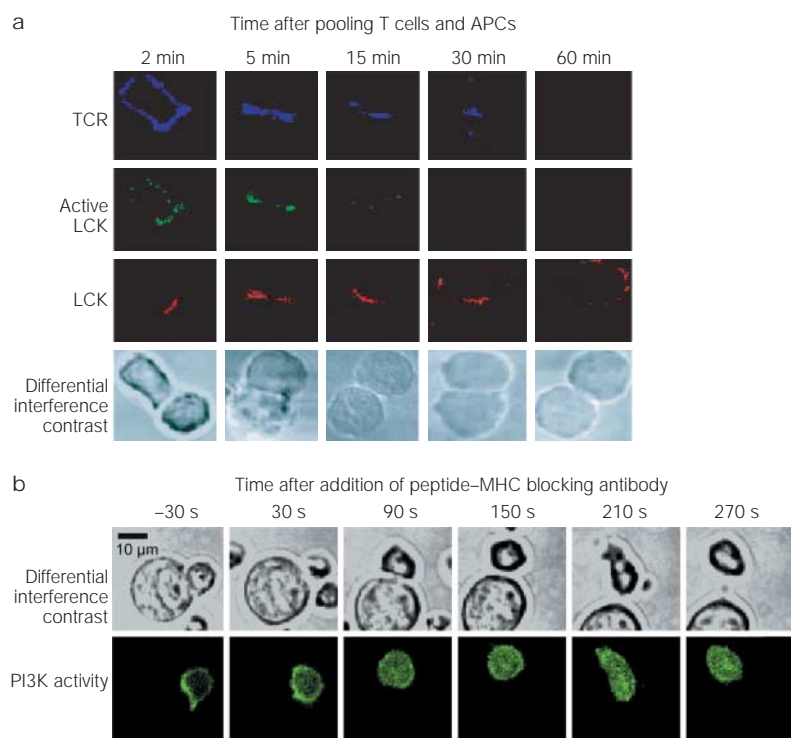


Figure 4 | Signalling in the immunological synapse. **a** | T-cell receptor (TCR) signalling precedes the formation of the immunological synapse⁵⁰. The tyrosine kinase LCK is activated at the periphery of the immunological synapse. TCR-transgenic splenic T cells were conjugated with splenic antigen-presenting cells (APCs) loaded with antigenic peptide. At the indicated time points, cells were fixed, permeabilized and stained with antibodies specific for the TCR (blue), LCK (red) and phosphorylated active LCK (green). The position of the cells is shown in the bottom row of images by differential interference contrast (DIC). Images reproduced from REF. 50 with permission from Science © 2003. **b** | Continuous TCR signalling that is required for synapse maintenance and full effector potential⁵². DIC shows that addition of antibodies specific for antigenic peptide–MHC class II complexes interferes with the T-cell–APC conjugate leading to the dissolution of the immunological synapse and T-cell detachment from the APC. Phosphatidylinositol 3-kinase (PI3K) activity gives an indication of signalling at the immunological synapse and can be assessed by the amount of PH(AKT)–YFP (pleckstrin homology domain of AKT, also known as protein kinase B (PKB), fused to yellow fluorescence protein) that is bound to the plasma membrane. The data in the lower panel of images shows the response of a 5-hour-old T-cell–APC conjugate to antibody treatment — PI3K signalling becomes less polarized over time, indicating that the synapse has dissolved. Numbers above the images refer to the time (seconds) after antibody addition. Image reproduced with permission from REF. 52 © (2003) Macmillan Magazines Ltd.

Within 45 seconds after contact between CD4⁺ T-cell blasts and B cells, activated ZAP70 was found in clusters that were evenly distributed over the entire interface³⁶. Surprisingly, activated ZAP70 disappeared three minutes after APC contact, but reappeared seven minutes after contact. The authors propose a ‘staging and resetting’ model that differentiates two phases of spatial–temporal activation, one before and one after SMAC buildup within the synapse. In conjugates of naive T cells and dendritic cells (DCs), active LCK and ZAP70 predominated in peripheral areas (high in CD4 and low in TCR)⁵⁰. Consistent with biochemical studies, Shaw and co-workers⁵⁰ detected active kinases only in the first 15 to 30 minutes after initial cell contact — that is, before the immunological synapse had acquired a mature phenotype (FIG. 4a). Clearly, TCR-proximal signalling events peak before the formation of a mature synapse and this renders models of synapse function in which its sole function is to facilitate signalling over a long period of time obsolete (see later). A recent study by Shaw, Chakraborty and co-workers⁵¹ aims to reconcile the rapid attenuation of TCR-proximal signalling after initial cell contact with a physiological function of a ‘mature’ immunological synapse. Here, the authors combine classical *in vitro* experimentation (biochemistry and immunohistochemistry) on the T-cell phenotype of Cd2-associated protein (Cd2ap)-deficient mice with a computational approach. Cd2ap-deficient T cells fail to efficiently degrade activated internalized TCR–CD3 complexes, maintain TCR-proximal signalling over an extended period of time, do not form cSMACs/pSMACs within the immunological synapse and are hypersensitive to challenge with antigen. By simulating the dynamics of receptor–ligand binding, signal transduction and protein movement with a Monte-Carlo algorithm, the authors are able to account for most of the main characteristics of the Cd2ap-deficient T cells. The authors conclude that the immunological synapse acts as a servo-controller that both boosts receptor triggering and attenuates strong signals⁵¹.

Synapse maintenance through TCR signals. T_H-cell–APC contacts are often sustained for more than ten hours. If not for the verification of antigenicity, what then might be the function of a highly ordered synapse and what are the forces that keep it in shape? Some answers came from a recent study that focused on the kinetics of the requirement of TCR-mediated signals for the activation of effector T_H cells⁵². The generation of two secondary messengers downstream of TCR ligation — the calcium signal and the induction of phosphatidylinositol 3-kinase (PI3K) activity — were recorded to boost the detection of TCR-derived signals. Despite the rapid removal of activated TCRs from the site of APC contact, CD4⁺ effector T cells continued to produce both messengers throughout the lifetime of the synapse (FIG. 4b). These signals depended on TCR ligation at all times. Enforced termination of signalling resulted in the immediate dissipation of an ordered synaptic architecture, often leading to the breakup of the conjugates. The production of interleukin-2 (IL-2)

and T-cell proliferation — two hallmarks of T-cell activation — were markedly impaired when antigen recognition was prematurely abrogated. A similar scenario holds up for naive CD4⁺ T cells, as shown by Schrum and Turka⁵³, in that prolonged, yet not necessarily uninterrupted, TCR stimulation had a cumulative effect on their proliferative capacity, resulting in up to nine cell divisions⁵³. By reversibly inhibiting the activity of LCK, Valitutti and colleagues⁵⁴ could show that TCR signalling maintains the synapse structure and that T cells combine signals received through the synapse even when signalling is temporarily interrupted. In conclusion, at least in the case of T_H cells, continuous TCR signalling is required for synapse maintenance and prolonged signalling for full effector and proliferative potential.

The litmus test: *in vivo* imaging. The standard tissue-culture assays that have been used in the experiments described so far are only a crude approximation of the complex environment that lymphocytes are exposed to *in vivo*. To what extent is the synaptic organization that is observed *ex vivo* relevant? Are such synapses formed *in vivo*?

The first *in vivo* evidence for the immunological synapse obtained by immunohistology of fixed lymph nodes showed that the TCRs on antigen-specific naive CD4⁺ T cells redistributed to the APC contact side only in response to antigen⁵⁵. Antigen-specific CD43–GFP-expressing T cells in intact explanted lymph nodes were visualized, and immunological synapses between these cells and DCs, from which CD43 was excluded, were observed. This had been shown before by the same group using a standard tissue-culture system⁵⁶.

When cultured in a three-dimensional collagen matrix cell-culture system, naive T cells had only transient interactions with APCs, yet still proliferated at levels that were achieved in static cultures, indicating that a stable synapse is not the only way to activate T cells⁵⁷. Two studies in lymph-node cultures give conflicting results with respect to this issue. In one study, T cells moved rapidly and formed few stable contacts in tissue perfused with 100% oxygen⁵⁸, whereas in another system providing atmospheric (that is, 20%) oxygen, T cells formed stable contacts⁵⁶. Together, these studies confirm in principle the existence of the immunological synapse *in vivo*. However, the duration of such cell contacts will probably be best determined in the intact living organism. A recent study on the migration behaviour of CD4⁺ T cells in inguinal lymph nodes of anaesthetized mice confirmed an overall high motility⁵⁹. T cells cycled between states of low and high mobility about every two minutes with an average velocity of 11 micrometer per minute, achieving peak velocities >25 micrometer per minute. There are, without doubt, marked differences in both the makeup and the lifetime of immune synapses, reflecting the *in vivo* situation and physiological state in which they are formed.

A plethora of synapses

We have defined a synapse as any flat interface established between T cells or their precursors and APCs, in which the APCs could be as diverse as thymic stroma, so-called professional APCs or target cells in the periphery. The exchange of antigenic information as conveyed through the interaction between TCR and peptide–MHC complexes is the common denominator of all synapses. However, as the meaning of this information depends on location in an individual and cell context, it is probably not surprising that both the composition of the immune synapse as well as the duration of T-cell–APC interaction can vary markedly. And although most of the pioneering studies used model systems comprised of primary T_H cells, T-cell lines and Jurkat T cells on the one hand and B-cell lines on the other hand, the field is now moving towards more specialized scenarios, and considerable ‘deviations from the rule’ are becoming increasingly evident.

Effector cells: T helper cells and CTLs. T_H cells usually remain conjugated with APCs for many hours (10–24 hours) and show continuously increased levels of intracellular calcium^{52,60} with ensuing changes in gene transcription that is detectable within several hours after initial APC contact⁴⁹ (FIG. 5). The secretion of translated IL-2 and other cytokines is directed towards the immune synapse where they can act most specifically on the recognized cell. T-cell proliferation begins about 24–48 hours after synapse formation and continues for several days, leading to a total of three to four cell divisions. As synapse maintenance requires continuous TCR-mediated signals⁵², we consider it probable that synapse termination results from their cessation. Despite an early peak in TCR-proximal tyrosine phosphorylation, which is followed by a rapid decline to undetectable levels, TCR signalling levels off completely after more than ten hours of cell contact. What attenuation mechanisms could be at work? TCR–CD3 complexes are rapidly removed from the cell surface when activated. However, they are subsequently replaced by newly synthesized receptors, and TCR cell-surface expression is fully restored after 24 hours⁵². So, although receptor internalization could have a decisive role in attenuating TCR-signalling events at an early stage, it seems less important for their complete termination at later stages when TCR cell-surface expression is restored. Negative regulation of TCR signalling through cytotoxic T lymphocyte antigen 4 (CTLA4) could provide the missing piece in this puzzle. CTLA4 competes with CD28 for binding CD80/CD86, and when bound to its ligand, it inhibits TCR-proximal signalling through the recruitment of the intracellular phosphatase SHP2 (SRC homology 2 (SH2)-domain-containing protein tyrosine phosphatase 2), which dephosphorylates activated CD3 subunits^{61,62}. Although absent from naive T cells, it is found in endosomal compartments in primed T cells yet becomes increasingly enriched within the synapse of conjugated T_H cells⁶³, either through direct transport of intracellular CTLA4 to the plasma membrane or by reduced internalization of newly synthesized CTLA4 molecules when T cells are activated (or by both mechanisms).

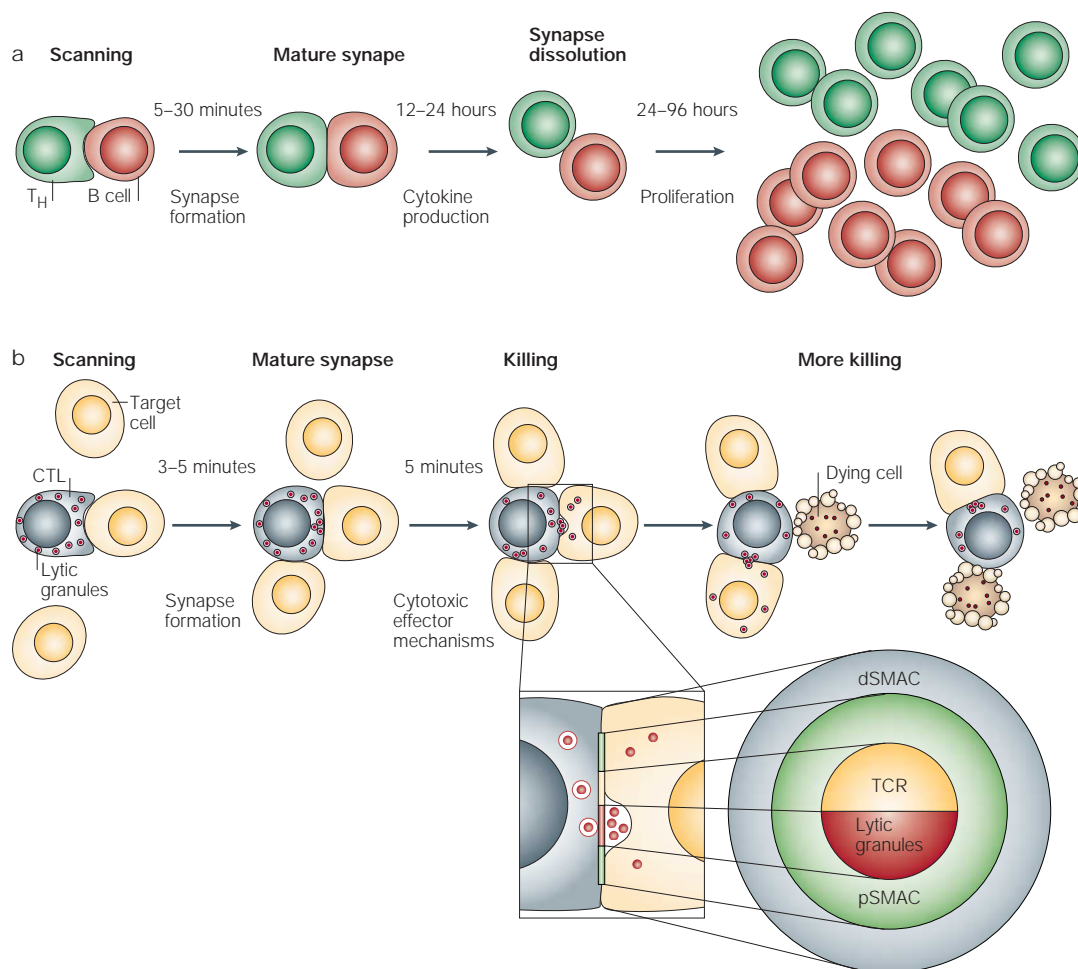


Figure 5 | Comparison between synapse formation in CD4⁺ T helper cells and cytotoxic T lymphocytes (CTLs).

a | CD4⁺ effector T cells form one mature synapse with B cells within 5–30 minutes after initial cell contact. This synapse lasts many hours during which cytokine production and release occurs and requires continuous T-cell receptor (TCR) signals for maintenance. Eventually conjugates break apart and cells undergo several divisions. **b** | CTLs form a rather transient synapse with their target cells and deliver their lethal hit within a few minutes. Lytic granules are rapidly transported to a region within the central region of the supra-molecular activation complex (cSMAC), which is devoid of TCRs. Whereas granule release takes place within minutes, target-cell blebbing occurs within 20–30 minutes, during which cells can still be loosely attached to one another.

A different scenario seems to occur for T-cell-mediated cytotoxicity, in which many of the events described earlier occur on an accelerated timescale and in which CD28-mediated co-stimulation seems to be less crucial. Whereas the contribution of the CTL compartment to the adaptive cytokine response is increasingly evident⁶⁴, the goal of the CTL–target cell synapse still seems to consist of the delivery of a lethal hit through the targeted release of cytolytic granules. These granules are rapidly focused underneath the synapse where they are in close proximity to the MTOC and then are shunted (probably on microtubule rails) through a specialized region within the cSMAC, which is devoid of TCRs⁶⁵. CTLs flux calcium for a comparatively short period of 2–20 minutes and cell detachment can occur shortly after initial cell contact. Primed CTLs are, similar to their T_H cell counterparts, highly sensitive to antigen, requiring only a few stimulatory peptide–MHC complexes for killing (M. Purbhoo *et al.*, unpublished observations).

Although target-cell death ensues in the order of hours, its irreversible initiation seems to require only minutes of CTL contact. In contrast to T_H cells, CTLs can form many synapses with several target cells at once⁶⁶, which causes an oscillating movement of the T cell MTOC between the target cell contact sites⁶⁷.

Thymic selection. Bousso *et al.*⁶⁸ were the first to use two-photon microscopy to monitor thymocyte behaviour in fetal organ cultures⁶⁸. This approach supported the visualization of individual cells, however, it does not yet provide a high enough resolution to image the spatial–temporal redistribution of cell-surface molecules in the area of contact. The specialized microenvironment of the thymus can now be mimicked *ex vivo* by pooling thymic stromal cells with thymocytes under conditions that support the growth of a multicellular ‘reaggregate’ entity in which positive and negative selection occurs readily and on an appropriate timescale. Considerably thinner than intact thymic lobes or fetal organ cultures, this system has

finally put the microscopic imaging of synapses between thymocytes and stromal cells within reach. Using this approach, Richie *et al.*⁶⁹ showed that conjugates formed rapidly when antigenic (negatively selecting) peptide was added; however, retrovirus-introduced CD3 ζ -GFP became enriched in the periphery and did not accumulate in the centre of the synapse as is the case when mature T_H cells interact with professional APCs. Although a lack of co-stimulation might in part explain this behaviour, this result illustrates the unique nature by which immature T cells process TCR-dependent stimuli. Further analysis of the contacts that occur between thymocytes and stromal cells is now being carried out and will help to elucidate the complex cell-surface biochemistry that ultimately results in negative as well as positive selection⁷⁰.

T cell-DC synapses. DCs are pivotal to adaptive immunity in that they either prime or anergize naive T cells in primary lymphatic organs, leading to either immunity or peripheral tolerance⁷¹. In response to various stimuli⁷², DCs upregulate their ability to process extracellular antigen and to provide co-stimulation^{73,74}, making them the most potent APCs that are known.

Mature DCs themselves become stimulated when recognized by naive T cells that are reactive to the antigen they present⁷⁵. In contrast to most other 'passive' APCs mentioned earlier, DCs undergo occasional fluxes in intracellular calcium and target intracellular MHC class II molecules along newly formed microtubules specifically to the synapse of the contacting antigen-specific T cell. How this cross-talk is achieved remains an open question. Signalling through ligated peptide-MHC complexes has been observed in B cells⁷⁶, and it is tempting to speculate that such a mechanism provides T-cell specificity to antigen-presenting mature DCs^{77,78}.

Conclusions and outlook

What is the function of the immune synapse? The initial reports of synapse structure and dynamics indicated that its function was to enhance and sustain signalling^{7,11,12}. Doubts about this interpretation were first raised by van der Merwe and Davis⁷⁹, who argued that it was equally plausible that the synapse structure functioned as a conduit for effector molecules, such as

cytokines or cytotoxic agents, that needed to be targeted specifically at the cell that is being recognized⁷⁹. This view gained some support from the work of Gunzer *et al.*⁵⁷ who saw only transient interactions between naive T cells and DCs in a collagen matrix system, and yet nearly full activation and proliferation⁵⁷. As TCR-proximal signalling is at its highest before a mature synapse with its typical 'bull's eye' appearance has formed, Shaw and co-workers⁵⁰ favour the idea that the immunological synapse is involved in TCR down-regulation and endocytosis⁵⁰, and propose, together with Chakraborty and colleagues⁵¹, that the synapse balances TCR signalling and degradation.

In light of our more recent findings⁵², we would suggest a similar, yet possibly more holistic, view in which although mature T-cell synapses are not strictly required for proliferation, they help to regulate the process⁵². We envision it as a platform that provides sufficient architectural complexity to accommodate regulatory mechanisms that are required to guide T-cell activity in accordance with its developmental stage, its range of functions, the nature of the APC involved, as well as both the quality and quantity of TCR ligands to be recognized. The fact that some synapses are long-lived, but others are of short duration, supports this notion.

A brave new imaging world. We live in exciting times in which continuous improvements in imaging technologies have led to amazing pictures of T cells at work and the beginning of a true integration of molecular and cellular dynamics following the behaviour of individual molecules. This might prove fruitful for the determination of the kinetics of receptor interactions as they occur between two cells. Sophisticated and computer-assisted electron microscopy might provide the missing link between conventional light microscopy and protein biochemistry. *In vivo* imaging could provide insights into pathogenic processes, such as the generation of autoimmunity, the inability to clear pathogen and tolerance towards cancer. And although much has been learnt in the field of T-cell-antigen recognition in the past five years through the use of imaging technologies, more can be expected from the next few years.

<p>1. Norcross, M. A. A synaptic basis for T-lymphocyte activation. <i>Ann. Immunol. (Paris)</i> 135D, 113-134 (1984).</p> <p>2. Paul, W. E. <i>et al.</i> Regulation of B-lymphocyte activation, proliferation, and differentiation. <i>Ann. NY Acad. Sci.</i> 505, 82-89 (1987).</p> <p>3. Spiegel, S., Kassis, S., Wilchek, M. & Fishman, P. H. Direct visualization of redistribution and capping of fluorescent gangliosides on lymphocytes. <i>J. Cell Biol.</i> 99, 1575-1581 (1984).</p> <p>4. Kupfer, A., Dennert, G. & Singer, S. J. Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets. <i>Proc. Natl Acad. Sci. USA</i> 80, 7224-7228 (1983).</p> <p>5. Kupfer, A. & Dennert, G. Reorientation of the microtubule-organizing center and the Golgi apparatus in cloned cytotoxic lymphocytes triggered by binding to lysable target cells. <i>J. Immunol.</i> 133, 2762-2766 (1984).</p> <p>6. Valitutti, S., Dessing, M., Aktories, K., Gallati, H. & Lanzavecchia, A. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy.</p>	<p>Role of T cell actin cytoskeleton. <i>J. Exp. Med.</i> 181, 577-584 (1995).</p> <p>7. Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. & Kupfer, A. Three-dimensional segregation of supramolecular activation clusters in T cells. <i>Nature</i> 395, 82-86 (1998).</p> <p>This landmark study showed the presence of supra-molecular activation complexes (SMACs) within the immune synapse.</p> <p>8. Donnadieu, E., Bismuth, G. & Trautmann, A. Antigen recognition by helper T cells elicits a sequence of distinct changes of their shape and intracellular calcium. <i>Curr. Biol.</i> 4, 584-595 (1994).</p> <p>9. Negulescu, P. A., Krasieva, T. B., Khan, A., Kerschbaum, H. H. & Cahalan, M. D. Polarity of T cell shape, motility, and sensitivity to antigen. <i>Immunity</i> 4, 421-430 (1996).</p> <p>10. Wulfig, C., Sjaastad, M. D. & Davis, M. M. Visualizing the dynamics of T cell activation: intracellular adhesion molecule 1 migrates rapidly to the T cell/B cell interface and acts to sustain calcium levels. <i>Proc. Natl Acad. Sci. USA</i> 95, 6302-6307 (1998).</p>	<p>11. Dustin, M. L. <i>et al.</i> A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts. <i>Cell</i> 94, 667-677 (1998).</p> <p>12. Grakoui, A. <i>et al.</i> The immunological synapse: a molecular machine controlling T cell activation. <i>Science</i> 285, 221-227 (1999).</p> <p>These authors were the first to use supported lipid bilayers as artificial antigen-presenting cells (APCs) to monitor the spatial-temporal redistribution of peptide-MHC complexes and intercellular adhesion molecule 1 (ICAM1) during the development of the immune synapse.</p> <p>13. Harding, C. V. & Unanue, E. R. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T-cell stimulation. <i>Nature</i> 346, 574-576 (1990).</p> <p>14. Demotz, S., Grey, H. M. & Sette, A. The minimal number of class II MHC-antigen complexes needed for T cell activation. <i>Science</i> 249, 1028-1030 (1990).</p> <p>15. Kimachi, K., Croft, M. & Grey, H. M. The minimal number of antigen-major histocompatibility complex class II complexes</p>
---	---	---

- required for activation of naive and primed T cells. *Eur. J. Immunol.* **27**, 3310–3317 (1997).
16. Irvine, D. J., Purbhoo, M. A., Krosgaard, M. & Davis, M. M. Direct observation of ligand recognition by T cells. *Nature* **419**, 845–849 (2002).
This paper provided direct evidence that effector T cells respond to a single stimulatory peptide–MHC complex and that CD4 is required for this degree of sensitivity.
17. Chang, W. R. *et al.* Crystal structure of R-phycoerythrin from *Polysiphonia urceolata* at 2.8 Å resolution. *J. Mol. Biol.* **262**, 721–731 (1996).
18. Peck, K., Stryer, L., Glazer, A. N. & Mathies, R. A. Single-molecule fluorescence detection: autocorrelation criterion and experimental realization with phycoerythrin. *Proc. Natl. Acad. Sci. USA* **86**, 4087–4091 (1989).
19. Feske, S., Giltiane, J., Dolmetsch, R., Staudt, L. M. & Rao, A. Gene regulation mediated by calcium signals in T lymphocytes. *Nature Immunol.* **2**, 316–324 (2001).
20. Boniface, J. J. *et al.* Initiation of signal transduction through the T cell receptor requires the multivalent engagement of peptide/MHC ligands. *Immunity* **9**, 459–466 (1998).
21. Cochran, J. R., Cameron, T. O. & Stern, L. J. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity* **12**, 241–250 (2000).
22. Randriamampita, C., Boulla, G., Revy, P., Lemaître, F. & Trautmann, A. T cell adhesion lowers the threshold for antigen detection. *Eur. J. Immunol.* **33**, 1215–1223 (2003).
23. Douce, M. A. *et al.* The β_1 and β_2 integrins promote T cell receptor-mediated cytotoxic T lymphocyte activation. *J. Biol. Chem.* **278**, 26983–26991 (2003).
24. Ge, Q. *et al.* Soluble peptide–MHC monomers cause activation of CD8⁺ T cells through transfer of the peptide to T cell MHC molecules. *Proc. Natl. Acad. Sci. USA* **99**, 13729–13734 (2002).
25. Kupfer, A. & Singer, S. J. Cell biology of cytotoxic and helper T cell functions: immunofluorescence microscopic studies of single cells and cell couples. *Annu. Rev. Immunol.* **7**, 309–371 (1989).
26. Wulffing, C. & Davis, M. M. A receptor/cytoskeletal movement triggered by co-stimulation during T cell activation. *Science* **282**, 2266–2269 (1998).
This paper indicated that co-stimulation is required for cytoskeleton-driven mass transport of lipids (and probably proteins) from the rear end of the T cell to the immune synapse.
27. Viola, A., Schroeder, S., Sakakibara, Y. & Lanzavecchia, A. T lymphocyte co-stimulation mediated by reorganization of membrane microdomains. *Science* **283**, 680–682 (1999).
28. Wulffing, C. *et al.* Co-stimulation and endogenous MHC ligands contribute to T cell recognition. *Nature Immunol.* **3**, 42–47 (2002).
29. Davis, S. J. & van der Merwe, P. A. The structure and ligand interactions of CD2: implications for T-cell function. *Immunol. Today* **17**, 177–187 (1996).
30. Shaw, A. S. & Dustin, M. L. Making the T cell receptor go the distance: a topological view of T cell activation. *Immunity* **6**, 361–369 (1997).
31. Wild, M. K. *et al.* Dependence of T cell antigen recognition on the dimensions of an accessory receptor–ligand complex. *J. Exp. Med.* **190**, 31–41 (1999).
32. Qi, S. Y., Groves, J. T. & Chakraborty, A. K. Synaptic pattern formation during cellular recognition. *Proc. Natl. Acad. Sci. USA* **98**, 6548–6553 (2001).
33. Burroughs, N. J. & Wulffing, C. Differential segregation in a cell–cell contact interface: the dynamics of the immunological synapse. *Biophys. J.* **83**, 1784–1796 (2002).
34. Johnson, K. G., Bromley, S. K., Dustin, M. L. & Thomas, M. L. A supramolecular basis for CD45 tyrosine phosphatase regulation in sustained T cell activation. *Proc. Natl. Acad. Sci. USA* **97**, 10138–10143 (2000).
35. Leupin, O., Zaru, R., Laroche, T., Muller, S. & Valitutti, S. Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes. *Curr. Biol.* **10**, 277–280 (2000).
36. Freiberg, B. A. *et al.* Staging and resetting T cell activation in SMACs. *Nature Immunol.* **3**, 911–917 (2002).
37. Sperling, A. I. *et al.* TCR signaling induces selective exclusion of CD43 from the T cell–antigen-presenting cell contact site. *J. Immunol.* **161**, 6459–6462 (1998).
38. Roumier, A. *et al.* The membrane-microfilament linker ezrin is involved in the formation of the immunological synapse and in T cell activation. *Immunity* **15**, 715–728 (2001).
39. Delon, J., Kaibuchi, K. & Germain, R. N. Exclusion of CD43 from the immunological synapse is mediated by phosphorylation-regulated relocation of the cytoskeletal adaptor moesin. *Immunity* **15**, 691–701 (2001).
40. Allenspach, E. J. *et al.* ERM-dependent movement of CD43 defines a novel protein complex distal to the immunological synapse. *Immunity* **15**, 739–750 (2001).
41. Savage, N. D. *et al.* Polar redistribution of the sialoglycoprotein CD43: implications for T cell function. *J. Immunol.* **168**, 3740–3746 (2002).
42. Khan, A. A., Bose, C., Yam, L. S., Soloski, M. J. & Rupp, F. Physiological regulation of the immunological synapse by agrin. *Science* **292**, 1681–1686 (2001).
43. Demetriou, M., Granovsky, M., Quaggin, S. & Dennis, J. W. Negative regulation of T-cell activation and autoimmunity by Mgat5 *n*-glycosylation. *Nature* **409**, 733–739 (2001).
44. Krummel, M. F., Sjaastad, M. D., Wulffing, C. & Davis, M. M. Differential clustering of CD4 and CD3 ζ during T cell recognition. *Science* **289**, 1349–1352 (2000).
This study showed that the T-cell receptor (TCR) and CD4 do not act together at all times during T-cell-antigen recognition, as they have a different distribution within the immune synapse at later stages.
45. Zal, T., Zal, M. A. & Gascoigne, N. R. Inhibition of T cell receptor–coreceptor interactions by antagonist ligands visualized by live FRET imaging of the T-hybridoma immunological synapse. *Immunity* **16**, 521–534 (2002).
46. Sloan-Lancaster, J. *et al.* ZAP-70 association with T cell receptor ζ (TCR ζ): fluorescence imaging of dynamic changes upon cellular stimulation. *J. Cell Biol.* **143**, 613–624 (1998).
47. Sloan-Lancaster, J. *et al.* Regulation of ZAP-70 intracellular localization: visualization with the green fluorescent protein. *J. Exp. Med.* **186**, 1713–1724 (1997).
48. Bunnell, S. C. *et al.* T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J. Cell Biol.* **158**, 1263–1275 (2002).
49. Ehrlich, L. I., Ebert, P. J., Krummel, M. F., Weiss, A. & Davis, M. M. Dynamics of p56lck translocation to the T cell immunological synapse following agonist and antagonist stimulation. *Immunity* **17**, 809–822 (2002).
50. Lee, K. H. *et al.* T cell receptor signaling precedes immunological synapse formation. *Science* **295**, 1539–1542 (2002).
This paper and reference 52 provide a relation between TCR signalling and the formation of the immune synapse.
51. Lee, K. H. *et al.* The immunological synapse balances TCR signaling and degradation. *Science* Sept (2003) doi: 10.1126/science.1086507.
52. Huppa, J. B., Gleimer, M., Sumen, C. & Davis, M. M. Continuous T cell receptor signaling required for synapse maintenance and full effector potential. *Nature Immunol.* **4**, 749–755 (2003).
53. Schrum, A. G. & Turka, L. A. The proliferative capacity of individual naive CD4⁺ T cells is amplified by prolonged T cell antigen receptor triggering. *J. Exp. Med.* **196**, 793–803 (2002).
54. Faroudi, M., Zaru, R., Paulet, P., Muller, S. & Valitutti, S. Cutting edge: T lymphocyte activation by repeated immunological synapse formation and intermittent signaling. *J. Immunol.* **171**, 1128–1132 (2003).
55. Reichert, P., Reinhardt, R. L., Ingulli, E. & Jenkins, M. K. Cutting edge: *in vivo* identification of TCR redistribution and polarized IL-2 production by naive CD4 T cells. *J. Immunol.* **166**, 4278–4281 (2001).
56. Stoll, S., Delon, J., Brotz, T. M. & Germain, R. N. Dynamic imaging of T cell–dendritic cell interactions in lymph nodes. *Science* **296**, 1873–1876 (2002).
57. Gunzer, M. *et al.* Antigen presentation in extracellular matrix: interactions of T cells with dendritic cells are dynamic, short lived, and sequential. *Immunity* **13**, 323–332 (2000).
58. Miller, M. J., Wei, S. H., Parker, I. & Cahalan, M. D. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* **296**, 1869–1873 (2002).
References 56, 57 and 58 are landmark imaging studies that provided the first insight into the dynamics of T-cell priming.
59. Miller, M. J., Wei, S. H., Cahalan, M. D. & Parker, I. Autonomous T cell trafficking examined *in vivo* with intravital two-photon microscopy. *Proc. Natl. Acad. Sci. USA* **100**, 2604–2609 (2003).
60. Iezzi, G., Karjalainen, K. & Lanzavecchia, A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* **8**, 89–95 (1998).
61. Lee, K. M. *et al.* Molecular basis of T cell inactivation by CTLA4. *Science* **282**, 2263–2266 (1998).
62. Egen, J. G., Kuhns, M. S. & Allison, J. P. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nature Immunol.* **3**, 611–618 (2002).
63. Egen, J. G. & Allison, J. P. Cytotoxic T lymphocyte antigen-4 accumulation in the immunological synapse is regulated by TCR signal strength. *Immunity* **16**, 23–35 (2002).
64. Harty, J. T., Tvinnereim, A. R. & White, D. W. CD8⁺ T cell effector mechanisms in resistance to infection. *Annu. Rev. Immunol.* **18**, 275–308 (2000).
65. Stinchcombe, J. C., Bossi, G., Booth, S. & Griffiths, G. M. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity* **15**, 751–761 (2001).
A study that provides detailed insight into the architecture of the cytotoxic T lymphocyte–target cell synapse.
66. McGavern, D. B., Christen, U. & Oldstone, M. B. Molecular anatomy of antigen-specific CD8⁺ T cell engagement and synapse formation *in vivo*. *Nature Immunol.* **3**, 918–925 (2002).
67. Kuhn, J. R. & Poenig, M. Dynamic polarization of the microtubule cytoskeleton during CTL-mediated killing. *Immunity* **16**, 111–121 (2002).
68. Bouso, P., Bhakta, N. R., Lewis, R. S. & Robey, E. Dynamics of thymocyte–stromal cell interactions visualized by two-photon microscopy. *Science* **296**, 1876–1880 (2002).
The first *in vivo* imaging study on thymic selection.
69. Riehle, L. I. *et al.* Imaging synapse formation during thymocyte selection: inability of CD3 ζ to form a stable central accumulation during negative selection. *Immunity* **16**, 595–606 (2002).
70. Hailman, E., Burack, W. R., Shaw, A. S., Dustin, M. L. & Allen, P. M. Immature CD4⁺CD8⁺ thymocytes form a multifocal immunological synapse with sustained tyrosine phosphorylation. *Immunity* **16**, 839–848 (2002).
71. Banachereau, J. & Steinman, R. M. Dendritic cells and the control of immunity. *Nature* **392**, 245–252 (1999).
72. Matzinger, P. The danger model: a renewed sense of self. *Science* **296**, 301–305 (2002).
73. Trombetta, E. S., Ebersold, M., Garrett, W., Pypaert, M. & Mellman, I. Activation of lysosomal function during dendritic cell maturation. *Science* **299**, 1400–1403 (2003).
74. Chow, A., Toomey, D., Garrett, W. & Mellman, I. Dendritic cell maturation triggers retrograde MHC class II transport from lysosomes to the plasma membrane. *Nature* **418**, 988–994 (2002).
75. Boes, M. *et al.* T-cell engagement of dendritic cells rapidly rearranges MHC class II transport. *Nature* **418**, 983–988 (2002).
Together with reference 74, these studies show that T-cell engagement of dendritic cells (DCs) triggers targeted transport of MHC class II molecules from lysosomes to the immune synapse. This means that signalling occurs in DCs in response to interactions with antigen-specific T cells.
76. Lang, P. *et al.* TCR-induced transmembrane signaling by peptide/MHC class II *in vivo* associated Ig- α / β dimers. *Science* **291**, 1537–1540 (2001).
77. McDonald, D. *et al.* Recruitment of HIV and its receptors to dendritic cell–T cell junctions. *Science* **300**, 1295–1297 (2003).
78. Geijtenbeek, T. B. *et al.* DC-SIGN, a dendritic cell-specific HIV-1 binding protein that enhances trans-infection of T cells. *Cell* **100**, 587–597 (2000).
79. Davis, S. J. & van der Merwe, P. A. The immunological synapse: required for T cell receptor signalling or directing T cell effector function? *Curr. Biol.* **11**, R289–R291 (2001).

Acknowledgements

We thank all members of the Davis and Chien Laboratory for thoughtful discussions and for maintaining a truly synergistic working environment. Work is supported by the Howard Hughes Medical Institute and the National Institutes of Health.

Competing interests statement

The authors declare that they have no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to: **LocusLink:** <http://www.ncbi.nlm.nih.gov/LocusLink/> | **Aggrin** | **CD2** | **Cd2ap** | **CD28** | **CD43** | **CD45** | **CD48** | **CTLA4** | **ICAM1** | **LFA1** | **MGAT5** | **NFAT** | **PKC- θ** | **SHP2** | **taln**

FURTHER INFORMATION

Alliance for cellular signalling: <http://cellularsignaling.org/>
Confocal microscopy: http://www.ai.mit.edu/people/minsky/papers/confocal_microscope.txt
Access to this interactive links box is free online.