Cytoskeletal polarization and redistribution of cell-surface molecules during T cell antigen recognition

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T cell antigen recognition is accompanied by cytoskeletal polarization towards the APC and large-scale redistribution of cell surface molecules into ‘supramolecular activation clusters’ (SMACs), forming an organized contact interface termed the ‘immunological synapse’ (IS). Molecules are arranged in the IS in a micrometer scale bull’s eye pattern with a central accumulation of TCR/peptide-MHC (the cSMAC) surrounded by a peripheral ring of adhesion molecules (the pSMAC). We propose that segregation of cell surface molecules on a much smaller scale initiates TCR triggering, which drives the formation of the IS by active transport processes. IS formation may function as a check-point for full T cell activation, integrating information on the presence and quality of TCR ligands and the nature and activation state of the APC.

Key words: T cell receptor / T cell antigen recognition / SMAC / immunological synapse / segregation

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Introduction

LYMPHOCYTES ARE PROPELLED through the blood as relatively undifferentiated spheres uniformly covered with short microvilli, but without obvious asymmetry or polarity. These cells also relatively non-adhesive, partly because integrins such as LFA-1 are immobilized by attachment to the actin cytoskeleton and maintained in a low avidity state. Activation of lymphocytes by, for example, chemotactic factors or ligation of antigen receptors results in increased adhesiveness, cytoskeletal polarization and the development of morphological asymmetry. It has long been recognized that cytoskeletal changes contribute to lymphocyte effector functions such as polarized cytokine secretion and target cell killing. Dramatic changes in cell-surface molecule distribution are also observed when the T lymphocytes recognise antigens on the antigen presenting cells (APCs). In this review we first describe the redistribution that occurs during T cell antigen recognition, placing it in the context of other cellular events such as changes in cell motility and morphology, increases in intracellular calcium, and polarization of the cytoskeleton. We then discuss in detail the molecular mechanisms underlying redistribution. Finally, we speculate on the functional significance of redistribution, and attempt to integrate the experimental data into a general model of T cell antigen recognition.

Observations

Snapshots of cytoskeletal polarization and cell-surface molecule redistribution

Studies performed over a decade ago showed that, following recognition of the antigen on target cells or APCs, T cells become polarized with respect to the target cell or APC (reviewed in ref 5). The microtubule organising centre (MTOC), which is usually...
found in the trailing edge of a migrating T cell, repositions itself between the T cell nucleus and the target cell.\textsuperscript{11} This is accompanied by, and is probably responsible for, the movement of the Golgi apparatus to the same side of the cell.\textsuperscript{11} There is also an increase in actin polymerization adjacent to the contact interface, and the actin-binding protein talin accumulates in this area.\textsuperscript{12} The T cell receptor and its CD3 signalling module (TCR/CD3), the TCR coreceptor CD4, and the adhesion receptor/ligand pair LFA-1/ICAM-1 all accumulate at the contact interface between CD4 T cells and APCs presenting specific antigens.\textsuperscript{5} Much more recently, Wulffing and Davis\textsuperscript{13,14} have reported evidence for a more generalized, cytoskeletonally-driven movement of cell surface molecules towards the T cell/APC contact interface.

Recently the redistribution of molecules within the contact interface has been visualized both in fixed T cell/APC conjugates\textsuperscript{7,8} as well as in live T cells interacting with planar, glass-supported lipid bilayers into which fluorescently-labelled, lipid-anchored molecules have been incorporated.\textsuperscript{10,15} These bilayers are capable of stimulating T cell proliferation, suggesting that they can function as surrogate APCs. While less physiological than APCs, the planar bilayer system has provided higher resolution images and the first quantitative and dynamic data on this redistribution. Both approaches reveal that cell-surface molecules segregate within T cell/APC contacts into distinct regions or clusters, referred to by Monks\textsuperscript{16} as supramolecular activation clusters (SMACs), thereby forming an organized interface which we refer to as an immunological synapse\textsuperscript{10} (Figure 1). The phrase immunological synapse (IS) was coined several years ago\textsuperscript{6} to draw attention to some of the similarities between these contact areas and neuronal synapses.\textsuperscript{16} We have extended the term to describe the characteristic molecular patterns formed in the process of physiological T cell activation.\textsuperscript{10}

IS formation passes through several stages (Figure 2), culminating in the ‘mature’ IS (Figure 1). In the mature IS the TCR/CD3 and several molecules involved in TCR triggering congregate at the centre of the interface.\textsuperscript{7,8,10} These include peptide–MHC, the accessory molecule CD28 and its ligand CD80, and cytoplasmic signalling molecules (e.g. lck, fyn, PKC-\(\theta\)). This central area has been termed the central cluster or cSMAC.\textsuperscript{8} A second group of molecules, including LFA-1, ICAM-1 and talin, form a ring surrounding the cSMAC, which has been termed the ‘outer adhesion ring’ or peripheral SMAC (pSMAC)\textsuperscript{7,8,10}. In the planar bilayer system\textsuperscript{10} the accessory molecule CD2 and its ligand CD48 appear to be confined to an ‘inner adhesion ring’. This surrounds the central zone but lies within the outer LFA-1 adhesion ring. Finally, CD43, a large, highly-abundant mucin-like molecule, is mostly excluded from the contact interface.\textsuperscript{9}

**Dynamics of polarization and redistribution**

Studies in live cells have provided insights into the timing and the sequence of events described above. T cell clones and hybridomas are highly mobile in cell culture, migrating at speeds of 4–10 \(\mu\)m/min.\textsuperscript{4,17} When they encounter surrogate APCs (usually cell lines transfected with suitable MHC molecules) that are not presenting specific antigens, they show no changes in motility or morphology.\textsuperscript{17,18} In contrast, when they encounter APCs presenting specific antigens they arrest, undergo shape changes, and expand their contact with the APC.\textsuperscript{17,18} T cells also migrate rapidly on artificial substrates coated with ICAM-1, but stop migrating when they encounter regions

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**Figure 1.** Structure of the mature immunological synapse. (a) Schematic view of the mature IS. The position of the IS is shown schematically above. The structure of the IS is shown below as viewed perpendicular to the contact interface. Not that some of the molecules are expressed on T cells and others on APCs. CD43 is excluded from the entire interface. (B) Fluorescent images revealing the position of the CD28/CD80 (left) and CD2/CD48 (right) complexes in the IS. The left image shows the positions of peptide–MHC (green), CD80 (red) and ICAM-1 (blue) at the interface between 3A9 T cells and planar bilayers. The right image shows the positions of peptide–MHC (green), CD48 (red) and ICAM-1 (blue) at interface between 2B4 T cells and planar bilayers. The CD28/CD80 complex colocalizes with the TCR/peptide–MHC whereas the CD2/CD48 complex segregates to an inner ring between TCR/peptide–MHC and LFA-1/ICAM-1. Data from Grakoui\textsuperscript{16} et al.

**Figure 2.** Steps in the formation of the IS. (A) Fluorescent images illustrating the dynamics of IS formation. T cells are brought into contact with planar lipid bilayers at \(t = 0\). The positions of engaged peptide–MHC (green) and engaged ICAM-1 (red) at the indicated times after initial contact are shown superimposed on an image of the contact area (white). Reproduced with permission from Grakoui\textsuperscript{16} et al.\textsuperscript{10} (B) The three stages of IS formation in planar lipid bilayer system. Reproduced with permission from Grakoui\textsuperscript{16} et al.\textsuperscript{10}
coated with ICAM-1 and specific peptide–MHC complex.

Together these data suggest that an encounter with specific antigens delivers a stop signal to migrating T cells, which serves to keep them associated with the APC that is presenting specific antigen. Naïve T cells are less mobile but also show rapid changes in morphology and motility upon contact with B cells presenting specific antigens. These changes in motility and morphology are followed in a variable time (seconds to minutes) later by increases, sometimes oscillating, in intracellular calcium concentration.

A consistent feature in all the above studies is that changes in T cell motility, morphology and intracellular calcium concentration are only seen if the APC or planar bilayers present specific antigens, indicating that they require TCR triggering. In contrast, when dendritic cells are used as APCs, morphological changes, conjugate formation, and increases in calcium are seen in the absence of antigens, even in the absence of MHC class II. This suggests that, unlike surrogate APCs or B cells, dendritic cells have the ability to engage and activate naïve T cells prior to any TCR engagement, perhaps through the expression of high levels of the CD28 ligands CD80 and CD86 or chemokines.

Technical innovations such as the use of green fluorescent protein chimeras and the planar bilayer system have made it possible to visualise the redistribution of cell surface molecules in live T cells. A recent study using primary T cells in the planar lipid bilayer system has revealed that the formation of the IS takes several minutes following initial contact and TCR triggering, and can be divided into at least three stages (Figure 2).

Following contact with planar bilayers presenting specific antigens, the T cells arrest and within 30 s a central zone containing the LFA-1 ligand ICAM-1 is formed. Analysis by interference reflectance microscopy indicated that an area of close contact surrounds the central zone. An unexpected finding was that clusters of engaged peptide–MHC first appeared in this outer close contact ring. Since no other accessory molecules were present in this system we (A.S. and M.L.D.) proposed that the cells were using the LFA-1/ICAM-1 interaction as an ‘anchor’ to force the membranes into close proximity. This raises the interesting possibility that active processes contribute to the membrane approximation that is necessary for the TCR to engage peptide–MHC complexes (see below).

Over the next 1.5–30 min the clusters of peptide–MHC in the outer ring moved towards the centre of the interface, forming a compact central cluster that is surrounded by a ring of ICAM-1 engaged to LFA-1. This organized interface, which is termed the ‘mature’ IS, is very similar to the pattern of SMACs described by Monks et al. The latter were visualized at the interface of T cells and specific antigens presenting APCs that had been fixed after 30 min of coculture. This transport of peptide–MHC is inhibited by cytochalasin D, suggesting that IS formation is dependent on the actin cytoskeleton.

Using peptide variants it was shown that the final density of peptide–MHC complexes in the central cluster correlated best with the half-life of the TCR/peptide–MHC interaction.

A final stage of IS formation was analyzed by fluorescent photobleaching recovery (FPR) experiments. This revealed that, after formation, the central cluster of the engaged peptide–MHC complexes does not exchange with free peptide–MHC outside the bleached area. The reasons for this are currently unclear, and it is not known whether the TCR is also stably associated with this central cluster. One possible explanation for this stability is that peptide–MHC complexes, possibly with bound TCR, oligomerise directly with each other, forming a two-dimensional lattice. A second possibility is that a complex network of associations between TCR/CD3, the co-receptors CD4 or CD8, accessory molecules, and their various extracellular and intracellular ligands, is stabilized through the cooperative effects of multiple weak interactions. How can this stability be reconciled with other data suggesting that TCR/peptide–MHC complexes are continuously forming and dissociating during T cell antigen recognition? One proposed explanation is that there is a high turnover of TCR/peptide–MHC complexes during the formation of the mature IS in the steps preceding stabilization of the central cluster.

A key finding to emerge from these dynamic studies is that TCR triggering is a very early event, and that changes in morphology, motility, calcium concentration and, in particular, IS formation all follow, and are dependent on initial TCR triggering. This raises three important questions that will be addressed in the remainder of this review. How does the initial TCR triggering occur? How does this triggering drive formation of the IS? Given that it is not required for initial TCR triggering, what is the purpose of IS formation?
Mechanisms

Overview

The processes contributing to the redistribution and segregation of cell surface molecules fall into two groups. Firstly there are passive mechanisms which are dependent on properties intrinsic to the molecules and their interactions. These include interactions with counter-receptors at the contact interface, physical dimensions, and partition into lipid rafts. Although not active in the sense of consuming cellular energy, these mechanisms do produce physical forces. Secondly, redistribution can be driven by active cellular processes, such as polarized secretion or remodelling of the cortical actin cytoskeleton.

Biophysical considerations

Molecular dimensions, membrane alignment and two-dimensional affinity

The molecular composition of the T cell surface has been intensively studied and is now reasonably well characterized.29 One striking observation30,31 is that these molecules vary enormously in size (Figure 3). The extracellular portions of the TCR and its ligand peptide–MHC are small (~ 7 nm) compared with several highly-abundant cell surface molecules such as CD43 (~ 45 nm) and CD45 (~ 28–50 nm), as well as important adhesion molecules such as integrins (~ 21 nm). It is conceivable that undulations in the plasma membrane could accommodate the size differences of interacting molecules. However, a second striking observation is that interactions between membrane-tethered adhesion molecules generate precise alignment of apposing membranes.15,32

Consequently, molecular interactions spanning different dimensions tend to segregate at the interface into distinct, homogenous zones.33 While the existence and significance of membrane alignment had been proposed earlier,34–36 it was demonstrated for the first time by a comparison of the solution affinity and the membrane (or two-dimensional) affinity of the CD2/CD58 interaction, using soluble and membrane-tethered forms of these molecules, respectively.15,32 The membrane affinity constant is two-dimensional because the concentration of membrane-tethered molecules is measured as surface density, a two-dimensional parameter (molecules/μm²). This comparison revealed that, by confining both interacting binding sites to a small volume (~ 5 nm high) between apposing membranes (the ‘confine-

ment region’), precise alignment of membranes enables the CD2/CD58 interaction, which has a low solution affinity (e.g. \( K_d \sim 2 \) μM), to achieve a high two-dimensional affinity (2D \( K_d \sim 1 \) molecule/μm²).15,32

The presence of large molecules at the cell–cell interface would be expected to act as a constraint on TCR engagement of peptide–MHC by preventing sufficiently close membrane approximation and membrane alignment.37 Indeed, T cell antigen recognition is enhanced when CD43 is absent from T cells.38 Notably, however, many T cell ‘accessory’ molecules which contribute to T cell antigen recognition are also small (Figure 3). These observation led to the suggestion30,39,40 that, in order for the TCR to engage peptide–MHC, molecules at the interface between T cells and APCs would have to segregate according to the size (Figure 4), with small accessory molecules contributing to the formation of ‘close-contact zones’, within which the inter-membrane separation distance (~ 15 nm) matched the dimensions of the TCR/peptide–MHC complex, and from which large molecules such as CD43, CD45 and integrins are excluded. Structural41–44 and mutagenesis45 studies of CD2 and its ligands CD48 and CD58 have shown that these molecules bind in a head-to-head orientation, forming a complex that spans the same dimensions (~ 14 nm) as the TCR/peptide–MHC complex. On this basis it was proposed that one function of CD2 is to facilitate the formation of close-contact zones, thereby enhancing TCR engagement of peptide–MHC.46,39,40 The importance of close membrane approximation is strongly supported by the recent observation that, whereas formation of the wild-type (~ 14 nm) CD2/CD48 complex strongly enhances TCR engagement of peptide–MHC, an elongated (~ 21 nm) CD2/CD48 complex is strongly inhibitory.37

Receptor lateral mobility

When two cells make contact, complementary surface receptors will usually need to move laterally within the plane of the membrane in order to encounter each other. This movement will depend on their lateral mobility,45 a term which refers collectively to two distinct properties; the proportion of the molecule that is mobile (the mobile fraction), and the diffusion rates of the mobile molecules within the plane of the membrane (the lateral diffusion coefficient).

There is currently only limited information on the lateral mobility of T cell surface molecules. Approxi-
mately 75% of the CD2 molecules expressed in Jurkat cells were shown to be mobile, similar to observations with other molecules. This agreed with the observation that a similar proportion of the CD2 accumulates in contact areas with CD58-bearing planar bilayers. In contrast, only 10–20% of the TCR...
on mature T cells is mobile. The reasons for this low mobility are not clear but may include cytoskeletal associations or diffusion barriers. The latter may be in the cytoplasm (e.g. cytoskeletal 'fences') or in the membrane itself (e.g. other membrane proteins or lipid rafts). This low mobility needs to be reconciled with experiments suggesting that up to 80% of cell surface TCR/CD3 engages peptide–MHC during antigen recognition. One possible explanation is that the immobile TCR/CD3 is actively transported to the contact interface over a long period. Alternatively, the T cell may form multiple contacts (simultaneous or sequential) with several APCs.

**Active processes**

There is compelling evidence that active processes contribute to the redistribution of cell surface molecules during T cell antigen recognition. Firstly, much of the observed redistribution requires and follows TCR triggering. Secondly, cytoskeletal inhibitors inhibit redistribution. Thirdly, a number of studies have shown that cross-linking of the TCR/CD3 complex with antibodies can trigger redistribution of TCR/CD3 ('capping') as well as other molecules ('co-capping') to one region (the 'cap') of the T cell surface. And finally, a cytoplasmic protein has been identified that interacts with the cytoplasmic domain of CD2 (CD2AP) and influences its distribution within a T cell/membrane interface.33

Two distinct transport processes have been implicated in the redistribution of cell-surface molecules; polarized secretion of cytoplasmic vesicles containing the molecules, and redistribution of molecules already at the surface.

**Polarized secretion**

Both CD8 T cells and CD4 T cells are capable of polarized secretion, in which cytoplasmic vesicles fuse to the plasma membrane at the contact interface with target cells or APCs. This allows the contents of these vesicles (e.g. perforin, granzymes, and cytokines) as well as the vesicle membranes to be directed to the interface. Recent data suggest that the T cell surface molecules Fast and CTLA-4 reside predominantly in cytoplasmic vesicular stores, and that these molecules are targeted to the site of TCR ligation by polarized secretion.

There is some evidence that lipid rafts and their associated molecules (see below) are concentrated in intracellular, presumably vesicular, stores in naïve T cells. Polarized secretion is known to target rafts to the apical membrane of epithelial cells. This raises the question as to whether lipid rafts and associated molecules are transported to the IS by polarized secretion.

**Movement of molecules on the cell surface**

Several lines of evidence implicate the cortical actin cytoskeleton (CAC) in the redistribution of surface molecules to the contact interface. Firstly, treatment of T cells with cytoskeletal inhibitors abrogates the.

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**Figure 3.** Dimensions of molecules involved in T cell antigen recognition. A schematic view of the dimensions of cell-surface molecules involved in T cell antigen recognition. Sizes are estimated from structural studies of these or related molecules with the exception of CD148. The size of CD148 (~ 55 nm) is estimated by adding 10 fibronectin type III domains strung end-to-end (≈ 40 nm) to an ~ 80 amino acid mucin-like region (≈ 15 nm).

**Figure 4.** The kinetic–segregation model of TCR triggering and immunological synapse formation. The redistribution of cell surface molecules (depicted as in Figure 3) in T cell activation. The green and red boxes on the left are enlarged views of the boxes on the right. (i) Before cell–cell contact the TCR/CD3 complex is subject to constitutive tyrosine phosphorylation and dephosphorylation. Dephosphorylation dominates, leading to a low steady-state level of tyrosine phosphorylation. Dephosphorylation raised the question as to whether lipid rafts and associated molecules are targeted to the IS by polarized secretion. (ii) Initial T cell contact with an APC leads to the formation of multiple close-contact zones within the contact area as a result of small-scale segregation of molecules. This is a passive process driven by size and ligand binding. Within these close-contact zones tyrosine phosphorylation is favoured because tyrosine phosphatases (e.g. CD45) are excluded, and tyrosine kinases are concentrated. (iii) Engagement by TCR of specific-peptide–MHC occurs within these close-contact zones and results in the trapping of the TCR/CD3 complex within a zone for a period, the length of which is determined primarily by the stability of the TCR/peptide–MHC complex. In the zone, TCR/CD3 is tyrosine-phosphorylated, initiating the multi-step signalling process required for triggering. (iv) A sufficiently stable TCR/peptide–MHC interaction (complex II) allows these signalling steps to be completed, leading to triggering. Unstable complexes (I and III) lead to no or partial triggering because, on leaving the close-contact zone, TCR/CD3, and any recruited signalling molecules, are re-exposed to high levels of tyrosine-phosphatase activity. (v) TCR triggering initiates active large-scale segregation of molecules, culminating in the formation of the mature IS. (vi) Steps (ii) to (v) are enhanced by ‘co-stimulatory signals’ such as chemokines or the expression of CD28 ligands on APCs. (vii) Sustained signalling through the IS results in full T cell activation.
redistribution of ICAM-1 and other molecules to the interface, and inhibits transport of peptide–MHC within the interface. Secondly, a large number of cell surface molecules which migrate towards and away from the interface have been shown to associate with the cytoskeleton or with linker molecules which themselves associate with the cytoskeleton (see below). Finally, ligation of the TCR leads to localized talin accumulation and actin polymerization.

How might the CAC organise the redistribution of cell surface molecules upon T cell activation? The CAC moves in a centripetal direction within the cellular processes that extend from cells, as a result of a combination of actin filament remodelling and myosin motor proteins. Following recognition, T cells extend analogous processes around the APC, forming a cup-shaped interface. Centripetal movement of the CAC within this ‘cup’ might move molecules coupled to the CAC, such as TCR/CD3, radially towards the centre of the contact area. How are some molecules (e.g. CD2 and LFA-1) prevented from moving into the centre and others (e.g. CD45) actively excluded? Possible explanations include the absence of links to the CAC, physical exclusion on the basis of size, and attachment to other cytoskeletal structures.

**Lipid rafts**

The plasma membrane of many cells, including T cells, contains microdomains which are enriched in glycosphingolipids and cholesterol, termed lipid rafts. Some T cell surface molecules important in TCR triggering (e.g. lck, fyn, LAT, CD48 and CD4) are preferentially associated with these structures. Others, most notably CD45, appear to be depleted from lipid rafts. This being so, the redistribution of lipid rafts could contribute to the segregation of cell surface molecules at the T cell/APC interface. Recent data suggest that triggering of the TCR can induce active redistribution of lipid rafts towards the site of TCR triggering. If cell-surface molecules that associate with lipid rafts, such as the GPI-anchored molecule CD48, bind to a ligand on APCs, this could provide a passive mechanism for the accumulation of rafts at the interface. The importance of lipid rafts in TCR triggering is highlighted by the finding that chemical disruption of rafts abrogates TCR triggering. Furthermore, mutations which disrupt the association with rafts of the essential signalling molecules LAT or lck also disrupt TCR triggering. The role of lipid rafts and LAT in TCR signalling are discussed in detail in the accompanying reviews by Janes et al and Zhang and Samelson, respectively.

**Linking TCR triggering to cytoskeletal polarization and receptor redistribution**

**Role of TCR triggering**

Anti-TCR antibodies can induce both re-orientation of the MTOC, polarized exocytosis and localized actin polymerization, suggesting that TCR triggering may be sufficient for these processes. However, an important caveat is that these experiments were performed in the presence of serum (a source of extracellular matrix proteins) or intact target cells, leaving open the possibility that integrin engagement is an additional requirement. As far as is known, TCR triggering is necessary for the re-orientation of the MTOC and Golgi apparatus towards the T cell/APC contact interface. In contrast, localized actin polymerization does not require TCR triggering. An interaction between LFA-1 on T cells and ICAM-1 on APCs is sufficient to induce cortical actin polymerization and talin accumulation at the contact site.

Anti-TCR antibodies can also induce redistribution of cell surface molecules, suggesting that TCR triggering is also sufficient for this to occur. It is unclear whether TCR triggering is absolutely necessary for active redistribution of cell surface molecules. The active redistribution described to date at T cell contact interfaces has always been preceded by TCR ligation. However, the various antigen-independent responses seen when T cells contact dendritic cells raise the possibility that TCR triggering may not be essential for redistribution at T cell/dendritic cell interfaces.

**Role of accessory molecules: a mechanism of co-stimulation?**

Recent studies have implicated accessory molecules in the active redistribution of T cell surface molecules. Wulfging and Davis showed that bulk redistribution of T cell surface molecules to the T cell/APC interface is inhibited when CD28/CD80 or LFA-1/ICAM-1 interactions are blocked. Viola et al found that the redistribution of lipid rafts to the area in contact with anti-CD3 antibody-coated beads is dramatically enhanced when the beads are also coated with anti-CD28 antibodies. These findings suggest that accessory molecules may enhance T cell activation by contributing to this redistribution process. The ability of the CD28/ligand interaction to activate Rho family...
GTPases (see below) independently of TCR triggering support this notion. As noted above, the LFA-1/ICAM-1 interaction can trigger changes in the CAC at the contact site. The finding that cytoskeletal polarization and large-scale receptor redistribution depend on TCR triggering, and are enhanced by engagement of accessory molecules such as CD28, suggests that IS formation is the point at which the T cell integrates information about the presence of the antigen on the APC and its activation state (e.g. expression of chemokines or CD28 ligands). Or, to rephrase in the terminology of the two-signal hypothesis of T cell activation, IS formation may integrate signal 1 (stimulus from antigen) with signal 2 (co-stimulus from the APC). This is supported by the finding that dendritic cells, the most effective APCs, are uniquely capable of adhering to and inducing antigen-independent responses in T cells.

Signalling pathways

Recently a tentative outline has emerged of the signal transduction pathways that couple TCR triggering to cytoskeletal polarization and receptor redistribution. Attention has focused on the Rho family of small GTP binding proteins (especially RhoA, Cdc42Hs and Rac1) since they have been shown to regulate cytoskeletal and vesicle transport processes in other cells. Using a T cell hybridoma, Stower et al. have shown that a dominant-negative mutant of Cdc42Hs inhibits actin and microtubule polarization towards the T cell/APC interface.

How does TCR triggering activate rho GTPases? The most likely candidate is the GTP/GDP exchange factor vav1, which activates Rac1 and has been reported to be essential for TCR-induced actin polymerization and capping. Vav1 is activated by tyrosine phosphorylation and/or by binding to polyphosphoinositide products generated following activation of phosphatidylinositol (PI) 3-kinase.

The mechanisms by which the rho GTPases regulate the cytoskeleton is not well-understood but is likely to involve effector proteins which regulate actin-binding proteins, such as the protein kinases ROCK and LIM-kinase. An effector protein already implicated in coupling Rho GTPases to the cytoskeleton in T cells is WASp, so named because it is defective in patients with Wiscott-Aldrich immunodeficiency syndrome. WASp interacts with Cdc42Hs and is required for capping induced by anti-CD3 antibodies, suggesting that it couples Cdc42Hs activation to the actin cytoskeleton.

Thus, the picture emerging is that TCR triggering activates Rho family GTPases through activation of proteins such as vav, and that Rho GTPases regulate various effector proteins, including protein kinases and WASp, which regulate in turn actin binding proteins. Direct links between vav and the actin and microtubule cytoskeleton have also been identified. Since the cytoskeleton and associated motor proteins control the active transport processes implicated in receptor redistribution, the vav/Rho pathway is likely to couple TCR triggering to cytoskeletal polarization and the redistribution of cell-surface molecules.

Linking the cytoskeleton to T cell surface molecules

For TCR-induced changes in the CAC to contribute the redistribution of cell-surface molecules, these molecules need to associate with the CAC. Linkages have been reported for LFA-1, which associates via talin, CD2, which associates with CD2AP and tubulin, the TCR, which associates via the CD3ζ chain, and CD45, which associates with the linker protein fodrin. Furthermore, members of the ezrin-radixin-moesin (ERM) family of proteins couple CD43, CD44, and ICAM-1, -2, and -3 to the CAC. With the exception of CD2AP (see below), the role of these linker molecules in surface molecule redistribution is not known. However, their diversity provides a possible explanation for the distinct localization of cell-surface molecules within the IS.

CD2AP

CD2AP was first identified as a molecule that binds to the cytoplasmic domain of CD2 and influences its distribution at the contact interface between a T cell and planar lipid bilayers. The finding that CD2/CD48 complexes occupy a special position in the IS is further evidence that CD2 is being actively moved at the interface, and it seems likely that this movement is also mediated by CD2AP.

A recent study on CD2AP-deficient mice suggests that this molecule has a wider role in lymphoid and non-lymphoid tissues. These mice die from complications of excessive leakage of serum proteins into the urine (nephrotic syndrome). This leakage appears to result from defects in the epithelial cell (podocyte) processes that contribute to the glomerular filtration barrier. Interestingly, CD2AP-deficient T cells show a substantial reduction in response to anti-CD3 antibodies, whereas CD2-deficient mice
are normal in this respect, suggesting that CD2AP plays a broader role in T cell function than CD2.

What might be the purpose of locating CD2/CD48 complexes in an inner ring surrounding the TCR/peptide–MHC-containing central zone? One intriguing possibility, consistent with a generalized role for CD2AP in forming filtration junctions, is that this ring of CD2/CD48 functions as a molecular filter within the IS keeping larger molecules out of the central zone in the face of centripetal actinomyosin-based transport processes.\textsuperscript{10,14}

**Functions**

What is the purpose of cytoskeletal polarization and redistribution of cell surface molecules? One probable role for cytoskeletal polarization is to facilitate the targeting by T cells of cytokines and the contents of lytic granules to the site of contact with APCs or target cells, respectively.\textsuperscript{5,55} As argued above, cytoskeletal polarization is also likely to contribute to the large-scale redistribution of cell surface molecules leading to the formation of the IS. Here we speculate on the role of IS formation in T cell activation, placing it in the context of a recently-proposed model of TCR triggering.\textsuperscript{39,40} It is important to re-emphasise here that the formation of the IS follows and depends on TCR triggering, indicating that it cannot be the mechanism of initial TCR triggering. Nevertheless IS formation does appear to be required for full T cell activation. What is the evidence for this? Firstly, in the few studies performed to date, IS formation correlated perfectly with full T cell activation.\textsuperscript{8,10} Secondly, disruption of the actin cytoskeleton\textsuperscript{80,87} or the regulation thereof,\textsuperscript{75–77} manipulations which would be expected to disrupt IS formation, leads to disruption of full T cell activation (proliferation and/or cytokine production) and negative selection. While not conclusive, these findings are consistent with an essential role for the IS in full T cell activation.

**Small-scale segregation provides a novel mechanism for signal transduction across membranes**

TCR triggering requires binding of the TCR to specific peptide presented on MHC class I or II molecules. One of the key, unresolved questions in T cell biology is how this binding generates a signal. In other words how does the T cell ‘know’ that a particular TCR has bound peptide–MHC? Based on the way they address this question, current models of TCR triggering can be divided into aggregation\textsuperscript{88,89} conformational change\textsuperscript{90,91} or segregation\textsuperscript{39,40,92,93} models. Segregation models have in common the proposal that peptide–MHC binding leads to triggering by holding the TCR/CD3 complex in a membrane environment in which tyrosine phosphatases have been segregated from tyrosine-phosphatases. Kinetic proof-reading,\textsuperscript{94} kinetic discrimination\textsuperscript{95} and serial triggering\textsuperscript{27} models do not fall neatly into the above classification because they do not address the central question of how binding transduces a signal. Instead they try to account for particular features of TCR triggering, such as the ability of the TCR to discriminate between very similar ligands\textsuperscript{94,95} and the ability of a few peptide–MHC complexes to engage many TCRs.\textsuperscript{27} Consequently they can and have been incorporated into aggregation, conformational change, or segregation models.

**Problems with aggregation and conformational change models**

Aggregation models postulate that peptide–MHC engagement leads to clustering or aggregation of TCR/CD3 complexes. At first glance this is an attractive notion since dimerization/aggregation is a well-established mechanism of signal transduction,\textsuperscript{96} and it is well known that aggregation of TCR/CD3, with either antibodies or multivalent peptide–MHC,\textsuperscript{97} will trigger TCRs. However, there are reasons for doubting that this is the physiological mechanism for TCR triggering.\textsuperscript{92} In systems in which dimerization/aggregation lead to signalling, the mechanism of multimerization is usually clear;\textsuperscript{36} a multivalent ligand (either a monomer with two binding sites or a multimer) binds two or more receptors, thereby bringing them into close proximity. In the case of the TCR the ligand, peptide–MHC, is monovalent, and present at an exceptionally low density, with as few as 10–100 molecules of a specific peptide–MHC on an entire cell. Thus, only a very small number of TCRs will be bound at any one time. Furthermore, each interaction is likely to be very short-lived, with a half-time of a few seconds.\textsuperscript{89} This suggests that simultaneous engagement of adjacent TCRs (i.e., aggregation) will be exceedingly rare. Furthermore, if TCR triggering depended on aggregation it should be very sensitive to the surface density of specific peptide–MHC, whereas the converse is true (see, e.g., refs 10,98). A refinement of the aggregation model, which attempts to address these problems, proposes that the binding of TCR to peptide–MHC generates a new binding site that enables TCR/peptide–MHC complexes to self-
associate. However, given the huge diversity in TCR and peptide–MHC structures, and the resulting diversity in the structure of TCR/peptide–MHC complexes, it is difficult to envisage how such binding sites could be conserved in all TCR/peptide–MHC complexes. A further difficulty is understanding how direct self-association occurs on the cell surface when each TCR is surrounded by several other molecules (e.g. CD3 subunits and a co-receptor), all of which are heavily glycosylated.

A variant of the aggregation model is that triggering is a consequence of heterodimerization of the TCR with its co-receptor (CD4 or CD8). While this may be sufficient for TCR triggering in certain circumstances, it cannot be the universal mechanism of triggering, since TCR triggering can occur in the complete absence of CD4 or CD8.

Conformational change models propose that peptide–MHC binding leads to a conformational change in the TCR, which is somehow transduced into the cell interior. This model requires that every TCR is able to undergo the same binding-induced conformational change in the face of huge diversity in the structure of the antigen binding sites and peptide–MHC, which would seem highly implausible. Furthermore, structural data available to date show that there are no conformational changes in the TCR induced by binding peptide–MHC except for adjustments within the antigen-binding site. Taking the latter data into account, Wiley et al. recently proposed a variant of the conformational change model based on studies of signalling through the erythropoietin receptor. In this model the TCR exists as a preformed dimer. When both TCRs in the dimer bind to peptide–MHC the two TCRs, reposition with respect to each other, and this repositioning is detected in some way. Unfortunately this model has the same drawback as the aggregation models when applied to TCR triggering (see above). A further difficulty is envisaging how the independent binding of two specific peptide–MHC complexes could change the relative positions of the two TCRs.

The kinetic-segregation model of TCR triggering

The arguments outlined above against conformational change or dimerization/aggregation models led to the proposal that an entirely novel mechanism, based on molecular segregation in contact areas, was responsible for TCR triggering. A related ‘topological model’, based on the implications of new data on two-dimensional affinity, subsequently converged on the same mechanism. These models emerged from several key observations described below.

Firstly, the TCR and T cell accessory molecules are much smaller than other abundant cell surface molecules, including CD43, CD45, and integrins (Figure 3). Taken together with the observation that membrane alignment is important for optimal binding of membrane-tethered molecules, this suggested that these molecules must segregate according to size at the contact interface.

Secondly, tyrosine phosphatase inhibitors can activate T cells, producing tyrosine phosphorylation patterns very similar to those seen during TCR triggering. Furthermore, an inhibitor of src kinases (PP1) rapidly and dramatically decreases the level of basal tyrosine phosphorylation in T cells. These findings show that the tyrosine kinases implicated in TCR triggering are constitutively active and under tonic inhibitory control by tyrosine phosphatases, and imply that altering the local balance between these two competing, constitutive processes would be sufficient to trigger intracellular signals.

Thirdly, one of the most active and abundant tyrosine phosphatases is the large cell surface molecule CD45. Furthermore, CD45 may associate directly or indirectly (via CD45-associated protein) with the TCR and its signalling apparatus. This suggested that, in addition to its generally accepted role maintaining lck in a primed state, increased CD45 may also tonically inhibit TCR triggering by dephosphorylating key tyrosine kinase substrates such as CD3ζ, ZAP-70 and lck itself. There is clear evidence that membrane-associated tyrosine phosphatases other than CD45 also reverse constitutively active tyrosine kinases. One possible candidate is CD148, which has recently been shown to inhibit TCR triggering. Interestingly, like CD45, CD148 is predicted to have a very large extracellular domain (Figure 3).

Finally, the TCR is able to discriminate between, and produce very different responses to, very similar ligands. This is difficult to explain purely on the basis of differences in affinity; all else being equal, a twofold change in affinity will produce a less than twofold change in the total number of engaged TCRs. In contrast, small kinetic differences can readily be amplified to produce very different biochemical responses.

These observations were synthesized into a new model of TCR triggering, which we now refer to as the kinetic–segregation model to emphasise its two key elements, molecular segregation and binding ki-
The model postulates the following sequence of events in TCR triggering (Figure 4).

When T cells encounter target cells or APCs, molecules at the contact interface will segregate according to size. Small molecules such as CD2, CD4, CD8 and CD28 will contribute to the formation of multiple, small close-contact zones, from which large molecules such as CD45, CD43, CD148 and LFA-1 are excluded.

As a result of this segregation, close-contact zones will be a ‘pro-signalling’ environment, relatively depleted of tyrosine phosphatases (such as CD45 and perhaps CD148) and enriched in tyrosine kinases such as lck. The close membrane approximation within the close-contact zone will facilitate TCR encounters with specific peptide–MHC. The binding of TCR to specific peptide–MHC will result in the TCR and its associated CD3 complex being held within a close contact zone, where they show a net increase in phosphorylation as a result of exposure to tyrosine kinases such as lck in the absence of tyrosine phosphatases such as CD45. It is important to emphasise here that ligation of a single TCR/CD3 can generate a signal (no dimerization or aggregation is required). Furthermore, tyrosine phosphorylation of TCR/CD3 is not strictly dependent on its localization to a ‘pro-signalling’ close contact zone, given that it occurs constitutively (see above). It is the downstream signalling events that are crucially dependent on localization to close-contact zones because this prolongs the half-life of tyrosine phosphorylated species by segregating the TCR/CD3 and associated molecules from tyrosine phosphatases. This tyrosine phosphorylation initiates a cascade of events including recruitment and activation of the tyrosine kinase ZAP-70 and phosphorylation and activation of ZAP-70 substrates. This sequence will be interrupted when the TCR/CD3 complex and associated molecules leaves such a close-contact zone. Because the TCR/CD3 is held within the contact zone through binding to peptide–MHC, tyrosine phosphorylation and TCR triggering will depend crucially on the half-life of the TCR/peptide–MHC interaction\textsuperscript{117} and a decrease in the half-life will lead to interruption of the cascade of signalling events.\textsuperscript{118,119}

To provide kinetic discrimination the model requires that initial close-contact zones be relatively small or short-lived so that inappropriately phosphorylated TCR/CD3 complexes do not have to diffuse far prior to their dephosphorylation by CD45 or other excluded phosphatases. This may be a key difference between the respective signalling roles of the initial close-contact zones and the IS, which forms over a much larger area. It also seems likely that there will be many such close-contact zones forming within the contact area between T cells and APCs. Unfortunately, given the limited resolution of the light microscope, the existence of such small close-contact zones remains speculative.

**Recent studies relevant to the kinetic–segregation model**

Since the kinetic–segregation model was first proposed a number of findings have provided support for key elements of the model. First, mice with reduced levels of CD45 expression show evidence of enhanced signalling through the TCR\textsuperscript{120} consistent with an inhibitory effect of CD45. Second, CD45 has been shown to inhibit the function of constitutively-active lck by dephosphorylating its substrates.\textsuperscript{121} Third, studies on planar bilayer systems have shown that engaged CD2 and LFA-1, which differ considerably in size (Figure 3), segregate in the contact interface independent of cytoskeletal interactions.\textsuperscript{33} This strongly supports the proposal that size differences drive the segregation of molecules within the contact interface. Fourth, it has been demonstrated that increasing the dimensions of an accessory molecule strongly inhibits TCR engagement.\textsuperscript{37} Fifth, a form of CD45 with a truncated extracellular domain has been shown to inhibit TCR triggering by antigens (C. Irles, A. Symons, F. Michel, P.A. van der Merwe, and O. Acuto, unpublished observations). Sixth, T cells are able to discriminate between peptide–MHC ligands with similar affinities but with differences in half-life for TCR binding.\textsuperscript{10,117} Finally, as with early TCR signalling events,\textsuperscript{117} the number of peptide–MHC complexes that accumulated in the central region of the IS correlated strongly with the half-life of the TCR binding.\textsuperscript{10} This provided the first direct evidence that IS formation is dictated by kinetics of the TCR/peptide–MHC interaction,\textsuperscript{10} most likely through the dependence of large-scale segregation on early TCR signalling events that are triggered, we suggest, by small-scale segregation.

TCR triggering is inhibited by cell-surface receptors such as CTLA-4 and Killer cell Immunoglobulin Receptors (KIRs), which associate with the cytoplasmic tyrosine phosphatases SHP-1 and SHP-2.\textsuperscript{122,123} Interestingly, the complexes formed by CTLA-4 and KIRs and their ligands (CD80/CD86 and MHC class I, respectively) are predicted to have dimensions compatible with their co-localization with the TCR in contact areas (Figure 3). Furthermore, CTLA-4 inhibits the earliest tyrosine phosphorylation events fol-
lowing TCR triggering. This suggests that inhibitory receptors may function by altering the balance between tyrosine phosphorylation and de-phosphorylation in the immediate vicinity of the TCR/CD3. As noted earlier, CTLA-4 is found predominantly in vesicular stores and is directed to the surface at the site of TCR triggering by polarized secretion. This suggests that CTLA-4 may be targeted to the centre of the IS as a means of modulating or terminating TCR signalling.

The kinetic–segregation model is compatible with evidence that lipid rafts play a crucial role in TCR triggering (see above). Lipid rafts may accumulate in or adjacent to the close-contact zones through either active or passive mechanisms, helping to contribute to the ‘pro-signalling’ environment by recruiting tyrosine kinases and other pro-signalling molecules and excluding CD45 (see accompanying review by Janes et al. Based on these recent data, new models of TCR triggering have been proposed which postulate that TCR binding to peptide–MHC brings the TCR and lipid rafts together. These ‘lipid raft models’ are similar in principle to the kinetic–segregation model in that they also propose that triggering is a consequence of the redistribution of the TCR into an environment enriched in tyrosine kinases and depleted of tyrosine phosphatases. These models may, however, need to be adjusted since recent evidence suggests that TCRs may be constitutively associated with rafts (see Janes et al). The main drawback of lipid raft models is that they do not suggest a clear mechanism, independent of aggregation or conformational change, by which engagement of the TCR with peptide–MHC either enhances its association with lipid rafts or promotes their aggregation.

**The immunological synapse as an activation checkpoint and signal integrator**

In addition to being structurally plausible, any model of TCR triggering has to explain a central paradox. The TCR detects peptide–MHC ligands with a wide range of affinities and surface densities but can nevertheless discriminate between peptide–MHC complexes with very subtle differences in binding properties. The ability of a given TCR to respond to a wide range of affinities is clearly illustrated by the ability of thymocytes to undergo positive and negative selection using the same TCR. More recently it has been shown that mature peripheral T cells also recognise and respond to self-peptide–MHC (reviewed in ref 126; see also refs 127,128). These same peripheral T cells require a much higher affinity TCR ligand in order to become fully activated. How is the TCR able to respond to ligands with such a wide range of affinities and yet retain the ability to discriminate between ligand which differ only slightly in their binding properties?

The kinetic–segregation model would allow very weak interactions to be detected because TCR/CD3 complexes need only be transiently detained in close-contact zones to become phosphorylated. Thus, tyrosine phosphorylation could occur even with very weak TCR binding to peptide–MHC. Perhaps this is sufficient to generate the signals required for positive selection in the thymus or survival in the periphery. The finding that ZAP-70 is constitutively associated with partially-phosphorylated TCR-ζ in peripheral T cells, but that this is reduced in T cells from mice lacking self-peptide–MHC and absent in T cells grown in culture, is consistent with continuous weak signalling through the TCR.

Because it would be inappropriate for peripheral T cells to be fully-activated by self-peptide–MHC, a mechanism must exist that enables a T cell to clearly distinguish between the signals resulting from weak versus strong self-peptide–MHC engagement. We suggest that the IS provides such a mechanism because it appears only to form when TCR binds peptide–MHC above a threshold affinity or half-life. Nevertheless the mechanisms that leads to IS formation are exquisitely sensitive since these structures form at very low surface densities of peptide–MHC. Taken together, these findings suggest that IS formation may function as a checkpoint for full T cell activation, facilitating discrimination between low affinity (presumably self-) peptide–MHC engagement and high affinity (presumably foreign-) peptide–MHC engagement.

Why might IS formation be required for full T cell activation? There are at least two possibilities. IS formation may lead to the generation of a unique signal. A candidate for such a signal is activated protein–kinase C-θ, which selectively accumulates in the central portion of the IS or cSMAC (see accompanying review by Kativar and Mochly-Rosen). A second possibility is that IS formation may be necessary for the prolonged (> 1 h) triggering necessary for full T cell activation.

If the IS is required for full T cell activation, it follows that any factor that contributes to IS formation will contribute to full T cell activation. Like full T cell activation, IS formation requires that the
TCR/peptide–MHC interaction affinity or half-life is above a threshold value. There is also intriguing evidence that ligation of accessory molecules such as CD28 and LFA-1 enhances some of the transport processes that lead to IS formation. Interactions with these molecules are regulated by soluble mediators (e.g. chemokines will activate LFA-1) and will depend on the activation or maturation state of the APC. Finally, there is evidence that dendritic cells are distinct from other APCs in their ability adhere to and evoke changes in naive T cells in the absence of and prior to antigen recognition. These data suggest that the formation of the IS serves to integrate information relating to the presence and quality of the antigen (e.g. surface density and binding properties) and information on the nature and activation state of the APC.

Based on these considerations we suggest the following integrated model of full T cell activation (Figure 4). Small scale-segregation, driven primarily by ligand binding and steric factors, leads to initial TCR triggering, by the mechanism outlined in the kinetic–segregation model. This is sufficient to stimulate certain T cell functions, the nature of which (positive selection or survival) depends on the developmental state of the T cell. Full activation, however, requires the formation of the IS, which involves active transport processes. IS formation requires a threshold level of triggering through the TCR and is influenced by the nature and activation state of the APC.

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