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Thirty-six views of T-cell recognition

Matthew Krummel, Christoph Wülfing†, Cenk Sumen and Mark M. Davis*

The Department of Microbiology and Immunology, and The Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305, USA

While much is known about the signalling pathways within lymphocytes that are triggered during activation, much less is known about how the various cell surface molecules on T cells initiate these events. To address this, we have focused on the primary interaction that drives T-cell activation, namely the binding of a particular T-cell receptor (TCR) to peptide–MHC ligands, and find a close correlation between biological activity and off-rate; that is, the most stimulatory TCR ligands have the slowest dissociation rates. In general, TCRs from multiple histocompatibility complex (MHC) class-II-restricted T cells have half-lives of 1–11 s at 25 °C, a much narrower range than found with antibodies and suggesting a strong selection for an optimum dissociation rate. TCR ligands with even faster dissociation rates tend to be antagonists. To observe the effects of these different ligands in their physiological setting, we made gene fusions of various molecules with green fluorescent protein (GFP), transfected them into the relevant lymphocytes, and observed their movements during T-cell recognition using multicolour video microscopy. We find that clustering of CD3ζ–GFP and CD4–GFP on the T cell occurs concomitantly or slightly before the first rise in calcium by the T cell, and that various GFP-labelled molecules on the B-cell side cluster shortly thereafter (ICAM-1, class II MHC, CD48), apparently driven by T-cell molecules. Most of this movement towards the interface is mediated by signals through the co-stimulatory receptors, CD28 and LFA-1, and involves myosin motors and the cortical actin cytoskeleton. Thus, we have proposed that the principal mechanism by which co-stimulation enhances T-cell responsiveness is by increasing the local density of T-cell activation molecules, their ligands and their attendant signalling apparatus. In collaboration with Michael Dustin and colleagues, we have also found that the formation and stability of the TCR-peptide–MHC cluster at the centre of the interaction cap between T and B cells is highly dependent on the dissociation rate of the TCR and its ligand. Thus, we are able to link this kinetic parameter to the formation of a cell surface structure that is linked to and probably causal with respect to T-cell activation.

Keywords: T-cell receptor; T cell; cell recognition; multiple histocompatibility complex; CD4; green fluorescent protein

1. INTRODUCTION

In 1830, the Japanese artist Hokusai began publishing his series of wood block prints entitled *Thirty-six views of Mt Fuji*. While the concept seems tedious and obsessive at first (and indeed it probably would have been at the hands of say, Andy Warhol), the execution was not, and these images remain well known and even iconic to this day (see www.csse.monash.edu.au/~jwb/ukiyoe/hokusai.html). Similarly, in modern biology it has often been productive to examine complex natural phenomena from a variety of experimental perspectives.

One such phenomenon that we have focused on is that of transient cell–cell recognition, as exemplified by T lymphocytes recognizing specific peptide–multiple histocompatibility complexes (MHC) on the surfaces of other cells. This process occurs continuously throughout the life of an individual and is vital for health. No evidence in support of this statement is more sobering than the fact that HIV infection, the subject of many of the papers in this issue, specifically devastates CD4 T cells and thereby leaves the victim open to many diseases that would ordinarily never be noticed. T cells as a distinct subset of lymphocytes have been a central object of study in immunology since their discovery four decades ago (Miller *et al.* 1961) and, as a consequence, all or most of the key surface molecules that they rely on for recognition and auxiliary activities are known. Thus, T cells represent an interesting (and clinically relevant) model system for studying how specific cell surface molecules mediate cell–cell recognition.

2. RECEPTORS AND LIGANDS INVOLVED IN αβ T-CELL RECOGNITION

A surprisingly large number of cell surface molecules appear to play important roles in T-cell recognition. These include the αβ form of T-cell receptors, an antibody-like heterodimer that is responsible for recognizing particular peptide–MHC complexes (on either class I or class II MHC molecules) on another cell. This is the key event in the decision to recognize one cell versus another.

* Author for correspondence (mdavis@cmgm.stanford.edu).
† Present address: Center for Immunology, UT Southwestern Medical Center, Dallas, TX 75390-9093, USA.

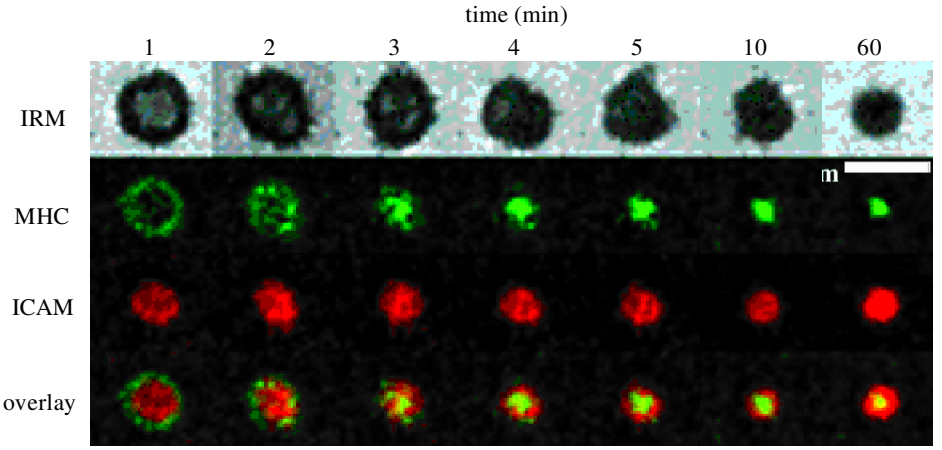

Figure 1. T-cell accumulation of MHC and ICAM and the formation of an immunological synapse. A representative primary T cell is shown interacting with a supported lipid bilayer containing two types of GPI-linked molecules: MHC (I-E^k) labelled with Oregon green dye and loaded with moth cytochrome *c* (88–103) peptide, and ICAM-1 labelled with Cy5 (red). The top panel uses interference reflection microscopy (IRM) to delineate regions of close T-cell membrane—bilayer apposition, which appear progressively darker as demonstrated by the outer ring in the T-cell contact at 1 min. The remaining panels show the redistribution of the labelled proteins in the contact area by fluorescence video microscopy over the course of 1 h. MHC is initially engaged in the tight outer edge of the contact, then actively accumulates into the centre of the synapse, where it forms a stable cluster. ICAM molecules exhibit a broader distribution throughout the interface, in some cases being excluded from the central MHC region, and serve as an adhesive fulcrum for T-cell attachment. From Grakoui *et al.* 1999.

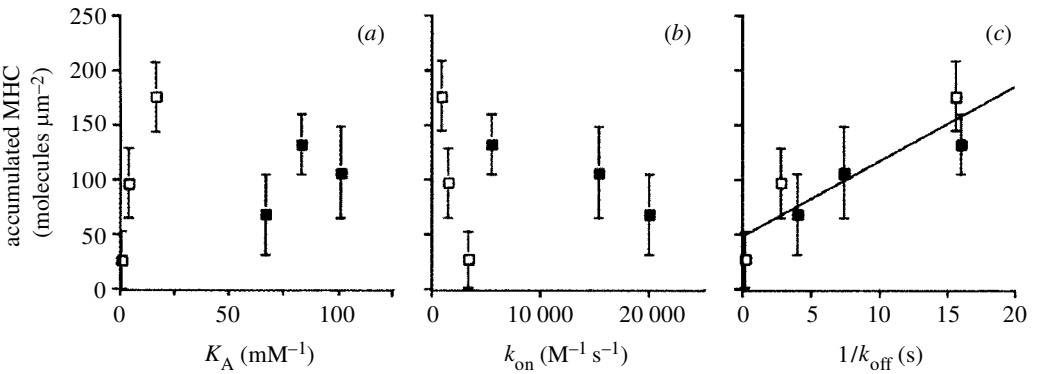

Figure 2. Correlation of MHC cluster density in T-cell contacts with the solution kinetic parameters of the TCR–MHC–peptide interaction. Pooling data from two different model antigen systems, Hb(64–76) (filled squares) and moth cytochrome *c* (88–103) (open squares), demonstrates that when the kinetic factors of affinity (*a*), association rate (*b*), and dissociation rate (*c*) are graphed versus MHC molecules per micrometre squared, only dissociation rate (plotted as the inverse) shows a close correlation to accumulated MHC. Thus, the half-life ($t_{1/2} = -\ln 2/k_{off}$) of the molecular interaction between TCRs and cognate MHC–peptide ligands determines the final MHC density in T-cell contacts. From Grakoui *et al.* 1999.

These heterodimers are always expressed together with the CD3 polypeptides, which are responsible for the signal transduction (Irving & Weiss 1991), as the TCR has no cytoplasmic domain with that capability itself. As few as ten to 100 of the correct peptide–MHC complexes are sufficient for recognition on the antigen-presenting cell (APC). As with other membrane molecules that recognize ligands on other cells, the affinity of a TCR for peptide–MHC is quite low, in the 10^{-4} to 10^{-6} m range (table 1). The off-rates range between 0.25 and 0.01 s^{-1} and in most systems an increasing off-rate correlates with loss of T-cell reactivity, even to the point where this can result in interference with an otherwise stimulatory signal (antagonism). Association rates are generally quite low at 500–10 000 M^{-1}s^{-1}, and recent thermodynamic evidence suggests that this may be due to a mechanism of binding in which somewhat disordered TCR-binding sites became more ordered upon ligand binding (Willcox *et al.* 1999; Boniface *et al.* 1999). This may correspond to the 'induced fit' mode of binding employed by DNA-recognition proteins, which also have to discriminate between a large number of chemically similar molecules (Boniface *et al.* 1999).

Other molecules on the T-cell surface act to facilitate recognition in ways that are not yet clear. These include LFA-1, which recognizes ICAM-1 on other cells but apparently only after it has been converted into a 'high affinity state' (still in the 1 μm range) by TCR engagement. The role of the LFA-1–ICAM-1 interaction has long been known to be important in T-cell adhesion, but it is also increasingly clear that it has a signalling role as well.

CD4 and CD8 are molecules specific to the helper or cytotoxic subsets of T cells, respectively, and bind class II or class I MHCs. The affinity of CD4 for class II MHC

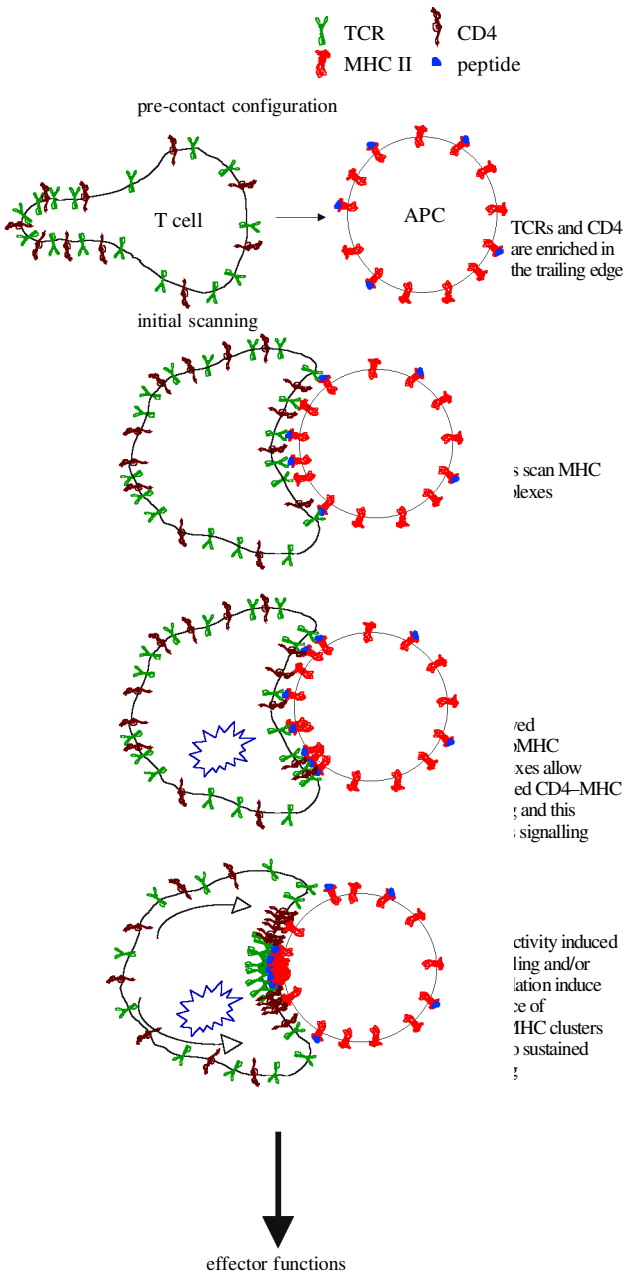

Figure 3. A model for checkpoints in T-cell activation. It is suggested that the progression to effector functions involves the ability of the TCR to (i) scan MHC complexes, then (ii) form long-lived TCR–pMHC complexes thereby recruiting CD4, which then (iii) initiates a cellular reorientation event stabilizing existing TCR complexes while recruiting more TCRs to prolong the response. The final configuration with TCRs highly localized into the middle of the interface is thus a result of traversing a number of checkpoints for TCR binding and pMHC recognition.

appears very weak and no direct binding measurements have been possible. For CD8, there are two conflicting data sets. In any event, the presence of CD4 or CD8 has the effect of amplifying the dose response of a given T cell by ten to 100 times. Other key molecules on the T-cell surface are CD28 and CTLA-4, both of which bind B7.1 (CD80) and B7.2 (CD86) on other cells and serve to respectively enhance or suppress T-cell activation.

CD28 ligation has a major 'co-stimulatory' effect on T-cell recognition, greatly amplifying activated T-cell responses and often being required for naive T-cell responses. A number of other T-cell molecules have also been implicated in assisting recognition, about which much less is known.

3. DISSECTING THE CHOREOGRAPHY OF T-CELL RECOGNITION: USE OF ARTIFICIAL MEMBRANES

How is it that these various molecules collaborate to allow T-cell recognition? For many years it has been known that clustering TCRs on T cells or Igs on B cells can artificially trigger activation. Subsequently, it has been shown that CD3ζ chimeras alone, when cross-linked, could induce T cells to become fully activated. This is analogous to other systems where receptor dimerization or multimerization initiates a signalling cascade (EGF). The fact that this was not the whole story was shown by the work of Monks *et al.* (1998), who used high resolution antibody staining of fixed T–B couples to show that TCR, LFA-1 and CD28 were not randomly distributed in the 'caps' at the interface but rather had a precise 'target' pattern, with TCR and CD28 occupying the 'bull-eye', LFA-1 enriched in a ring around it on the T cell, and a minor image of ligands on the B-cell side (e.g. MHC and B7 in the middle, ICAM-1 outside). This striking pattern has been dubbed an 'immunological synapse' and is associated with strong T-cell stimulation.

In parallel with this work have been the efforts of Michael Dustin and his colleagues to develop a dynamic system to study the movements of membrane molecules during T-cell recognition (Dustin *et al.* 1998). Here, surface molecules that have been engineered to have lipid tails (so that they are laterally mobile) are labelled with a particular fluorophore and then placed in a lipid bilayer on a glass slide. T cells are then deposited on this slide and the movement of the different molecules is observed by time-lapse video microscopy. The flat surface of the membrane on the slide ensures a flat interface and high resolution. As shown in figure 1, when an MHC–peptide complex is labelled with fluorescein (green) and ICAM-1 is labelled with rhodamine (red), a T cell recognizing these molecules quickly clusters them underneath itself in a characteristic 'synapse' pattern, with the MHC in the interior and the ICAM-1 in a (largely) concentric ring around it. This exactly parallels the findings of Monks *et al.* (1998), but has the added advantage of showing the dynamics of the system as well as the fate of individual T cells. A particularly striking finding of this work (Grakoui *et al.* 1999) is that the density of MHC clustering is proportional to the monomeric half-life of the particular peptide–MHC complex with the TCR (as shown in figure 2*c*)—thus establishing a link between the kinetic measurements summarized in table 1 with movements of populations of molecules on cell surfaces. The data in figure 2 also firmly establish the importance of dissociation rate versus affinity or association rate in determining the density of MHC clustering. Thus the stability of TCR binding to peptide–MHC is critical to synapse formation and this in turn is linked to a robust T-cell response, presumably as an increased density of ligand promotes a higher concentration of TCR–CD3, which in turn potentiates internal signalling.

Table 1. *Kinetic parameters of T-cell surface molecules: ligand binding measured by surface plasmon resonance*

T-cell molecule	ligand	species	K_D (μM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	references
TCR	MHC–peptide[a]	mouse	90–2	11 000–850	0.25–0.01	Davis *et al*. 1998; Savage *et al*. 1999
TCR	MHC-SAg (SEA)	mouse	2.3	4800	0.01	Redpath *et al*. 1999
CD8αα	MHCI	human	200–30	100 000–1200	18–0.05	Wyer *et al*. 1999; Garcia *et al*. 1996
CD28	CD80 (B7-1)	human	4	660 000	1.6	Van der Merwe *et al*. 1997
CTLA-4	CD80 (B7-1)	human	0.4	940 000	0.43	Van der Merwe *et al*. 1997
CD2	CD48	rat	90–60	100 000	6	Van der Merwe *et al*. 1993
CD2	CD58 (LFA-3)	human	22–9	400 000	4	Van der Merwe *et al*. 1994
CD62L	GlyCAM	human	105	100 000	10	Nicholson *et al*. 1998

[a] Range includes agonist peptides only for both MHC class I and II.

4. LABELLING T CELLS DIRECTLY: BEADS AND GREEN FLUORESCENT PROTEINS

While extremely useful in defining and following the molecules on APCs that are the ligands for T-cell recognition, this type of system is not as well suited for studying T-cell molecules. This is because it is the T cell that is the active partner here, with the movements of its molecules driving the action of molecules on the APCs. The T-cell molecules are also linked to the cytoskeleton in ways that are not well defined and so it is not possible to reproduce this on a glass slide. Therefore, we have labelled intact T cells in two different ways: (i) by attaching large beads (4–5 μm), either via antibodies to cell surface molecules that are neutral with respect to activation or through biotinylated lipids and streptavidin-coated beads (Wülfing & Davis 1998), or (ii) using green fluorescent protein (GFP) fusions to the cytoplasmic regions of various cell surface proteins (Wülfing *et al*. 1998; Krummel *et al*. 2000). Using the first procedure, we have been able to show that there is a distinct cell surface transport mechanism on T cells that is linked to the cytoskeleton and is triggered through the 'co-stimulatory' receptors CD28 and LFA-1. This effect results in the beads bound to the anterior of the T cell translocating to the interface with an APC, beginning at about 4 min after the first calcium flux and probably continuing throughout the first 30–60 min of recognition. We believe that this mechanism serves to deliver T-cell surface molecules and their subcellular components to the synapse region and is likely to be the chief reason that molecules accumulate there much more quickly than one would expect based on passive diffusion. It may also explain why cytoskeletal inhibitors such as cytocholasin D interfere so profoundly with T-cell recognition (and yet have no effect on APC; Wülfing *et al*. 1998). Similar observations regarding a co-stimulation-driven transport mechanism have been reported by Viola *et al*. (1999), who have linked it to lipid 'raft' movement using a cholera-toxin-staining reagent. Thus, it may be that there is a cytoskeletal linkage to membrane rafts that transports them (and their cargo) to the interface. In any event, the transport of key recognition molecules to the T-cell–APC interface would elevate the concentration of such molecules and thereby potentiate T-cell activation. As noted elsewhere (Cornall *et al*. 1998), even modest increases in the number of signalling molecules can have a significant effect on the sensitivity of lymphocyte activation. In light of this data, we have proposed that the entire enhancement effect seen with co-stimulation in T cells may be due to this type of effect (Wülfing & Davis 1998).

The second and more powerful approach to the issue of how to follow the dynamics of molecules on T cells involves the use of GFP fusions. This method has proven widely useful in a great number of biological applications previously (Tsien 1998), but it can be argued that its most significant impact could be in the understanding of cell surface molecules. The chemistry of soluble proteins can be readily studied in solution (at least in theory), whereas the dynamics of cell surface proteins is not easily studied or can only be studied to the sort of first approximation illustrated by the data in table 1. These measurements are necessary in order to understand the monomeric interaction dynamic but are inherently unable to provide information regarding the effects of movement in a bilayer–raft, the effects of polyvalency or the cooperativity between different types of molecules (either directly or through their respective signalling pathways), or the consequences of spatial segregation at the interface (synapse formation). GFP fusions allow us to study individual molecules at 37 °C in their native context. Our early efforts in this regard showed that ICAM-1–GFP on B cell APCs clustered rapidly in the synapse with a T cell, beginning about 20 s after the first rise in intracellular free calcium (which we can monitor simultaneously). This clustering of ICAM-1 did not occur in cells that lacked co-stimulatory ligands and served to potentiate calcium signalling and, by inference, the T-cell response (Wülfing *et al*. 1998).

Our most recent GFP work has focused on T-cell molecules, where we have labelled CD3ζ (the most important of the CD3 chains with respect to signalling), TCRα and CD4. In none of these cases did the GFP fusion (to the

cytoplasmic domain) have any noticeable effect on T-cell activation or signal transduction. To overcome the problem of the non-planar nature of the T-cell–APC couple, we have also constructed a video fluorescence microscopy apparatus, which uses a moveable objective to collect optical sections (20–30 at 0.5–1.0 μm thick) through the cell couples, allowing us to view the interface in three dimensions (Krummel *et al.* 2000). Using this system, we have been able to show that TCR–CD3 clusters rapidly to the synapse, coincident with the first calcium flux, and either remains there if activation is stable or dissipates and re-forms if it is not. Furthermore, CD4 GFP initially co-localizes to the centre of the interface but then is dispersed to the outer edges soon afterwards. This runs contrary to the notion of CD4 as a co-receptor that serves to stabilize TCR–peptide–MHC interactions (Janeway 1992) and instead suggests that it may be acting to potentiate only the early phases of recognition. CD4 GFP lacking its cytoplasmic domain also behaves in the same way, so this pattern of movement could not be orchestrated by a cytoskeletal linkage to CD4 but instead might be the result of some type of physical exclusion occurring at the centre of the synapse (Krummel *et al.* 2000).

5. CONCLUSION

A summary of what we have learned thus far is schematized in figure 3. Here we see a T-cell blast (that is, one that had been in contact with ligand a week or so beforehand) encountering an APC of the 'professional' variety (B cells, dendritic cells or macrophages), which possesses co-stimulatory ligands. If an initial survey of the APC's membrane turns up a sufficient number (*ca.* ten to 100) of peptide–MHC complexes that can stably bind to its TCRs, small clusters of TCR–CD3–CD4 can form perhaps purely by chemical attraction, as seen in solution by Reich *et al.* (1997); and this may allow the first release of calcium from intracellular stores. It also leads to synapse formation, in which much larger clusters of TCR–CD3 form, probably driven by the co-stimulatory transport mechanism described above, which also serves to cluster MHC molecules on the APC side by an 'effect'. By means and for reasons that are unknown, synapse formation is also accompanied by the segregation of LFA-1 and other molecules to the outer ring area, where they are joined by CD4. The establishment and maintenance of such a synapse for minutes or more correlates with a robust (and irreversible) commitment to T-cell activation and the release of cytokines in the case of T helper cells (parallel studies on cytotoxic T cells have not yet been done, but will presumably yield similar results).

Why is synapse formation important? We think that T-cell recognition is an inherently 'noisy' process with a large number of false starts. While this noisiness is probably a consequence of the extreme sensitivity of T cells to minute quantities of a particular antigen, this would make the danger of autoimmunity much more probable. Thus, synapse formation may serve as a proofreading process in which a larger area of the APC membrane is surveyed for ligand, and only if additional 'examples' are found does the T cell commit to the 'effector' phrase characteristic of full activation.

Clearly we are just at the beginning of a new appreciation of how cell surface molecules on T cells scan cells in the body and arrive at a decision as to whether or not to achieve full activation. This decision depends on cytoskeleton and signalling pathways inside the cell as well as the ligands available to the T-cell surface molecules. It is also dependent on synapse formation, although the purpose of this particular configuration of cell surface molecules is not clear, beyond the clustering of TCR–CD3 complexes and the likely cascade of tyrosine kinase activity that would ensue. Another mystery concerns the events leading up to the initial activation events—or rather, what distinguishes these from the random 'noise' that a T cell encounters from the large number of peptide–MHCs on various cell surfaces that it would encounter every working day. It is also mysterious how co-stimulatory signals trigger the movement of cell surface molecules and what different signals through LFA-1 and CD28 are doing (as they are synergistic they must be different in some way). Thus we have many phenomena operating in the same time-frame that are linked to the various cell surface molecules and are somehow integrated to contribute to T-cell recognition. Clearly more 'views' of greater molecular clarity will be needed to properly understand how this works, but much progress has been made in the last few years and it may not be long before we can grasp the main elements and their relationships.

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