Immunity, Vol. 9, 459–466, October, 1998, Copyright ©1998 by Cell Press

Initiation of Signal Transduction through the T Cell Receptor Requires the Multivalent Engagement of Peptide/MHC Ligands

J. Jay Boniface,[1,5] Joshua D. Rabinowitz,[3] Christoph Wülfing,[2] Johannes Hampl,[1,6] Ziv Reich,[1] John D. Altman,[1,7] Ronald M. Kantor,[2] Craig Beeson,[3,8] Harden M. McConnell,[3] and Mark M. Davis[1,2,4]

[1]Department of Microbiology and Immunology
[2]Howard Hughes Medical Institute
[3]Department of Chemistry
Stanford University
Stanford, California 94305

Summary

While much is known about intracellular signaling events in T cells when T cell receptors (TCRs) are engaged, the mechanism by which signaling is initiated is unclear. We have constructed defined oligomers of soluble antigen-major histocompatibility complex (MHC) molecules, the natural ligands for the TCR. Using these to stimulate specific T cells in vitro, we find that agonist peptide/MHC ligands are nonstimulatory as monomers and minimally stimulatory as dimers. Similarly, a partial-agonist ligand is very weakly active as a tetramer. In contrast, trimeric or tetrameric agonist ligands that engage multiple TCRs for a sustained duration are potent stimuli. Ligand-driven formation of TCR clusters seems required for effective activation and helps to explain the specificity and sensitivity of T cells.

Introduction

In general, upon ligand binding, a cell surface receptor initiates a signaling cascade inside of a cell by either undergoing a conformational change or by clustering. While it has been clear for many years that cross-linking of TCR complexes can cause T cell activation (Kappler et al., 1983; Kaye et al., 1983; Meuer et al., 1983; Watts, 1988; Symer et al., 1992; Kolanus et al., 1993; Spencer et al., 1993), it has remained controversial whether the binding of a single TCR by its natural ligand is sufficient as well (Karjalainen, 1994; Takahama et al., 1994). Moreover, if receptor clustering is required, the number of TCR molecules that are necessary to make a productive signaling unit is unclear. In the case of TCRs, distinguishing between these two signaling mechanisms has been daunting for several reasons. First, their natural ligands are themselves membrane-associated molecules (MHC) that are complexed with antigenic peptides (Ag). Second, the affinities of TCRs for Ag/MHC complexes are generally low (with K_Ds from 10^{-7} to 10^{-4} M) (reviewed in Davis et al., 1998). Third, T cells contain coreceptors (either CD4 on helper T cells or CD8 on cytotoxic T cells) that bind MHC molecules independent of antigen and contribute to signaling by bringing the protein Tyr kinase $p56^{lck}$ to the site of TCR engagement (Shaw et al., 1990; Turner et al., 1990). Lastly, the TCR complex, which contains no intrinsic enzymatic activity, contains three other apparent dimers, CD3γε, CD3δε, and a ζ homodimer, that possess sites for Tyr phosphorylation and substrate binding but organize in an unknown manner with respect to each other and to the TCR itself (reviewed in Alberola-Ila et al., 1997).

[4]To whom correspondence should be addressed (e-mail: mdavis@cmgm.stanford.edu).
[5]Present address: Eos Biotechnology, Inc., 225A Gateway Boulevard, South San Francisco, CA 94080.
[6]Present address: SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025.
[7]Present address: Department of Microbiology and Immunology, Emory University, 3119 Rollins Research Center, 1510 Clifton Road, Atlanta, GA 30322.
[8]Present address: Department of Chemistry, University of Washington, Box 351700, Seattle, WA 98195–1700.

Results

BIAcore Characterization of MHC Oligomers

To address the molecular mechanism of signaling through the TCR, we have constructed oligomers of a soluble form of a singly C-terminally biotinylated murine class II MHC molecule, IE^k (Altman et al., 1993; Gütgemann et al., 1998), by linking it at different valencies to a streptavidin molecule (Altman et al., 1996). Moth cytochrome c peptide (MCC) bound to IE^k is recognized by the well characterized T cell hybridoma, 2B4 (Hedrick et al., 1982; Samelson et al., 1983). MCC/IE^k-only (no streptavidin) and MCC/IE^k monomer (one MCC/IE^k per streptavidin) bind a soluble form of the 2B4 TCR with similarly transient kinetics (Figure 1A) (Davis et al., 1998). As expected, MCC/IE^k dimers and tetramers bind much more tightly to the TCR surface with dissociation rates approximately 10- and 170-fold slower, respectively, indicating that each MCC/IE^k molecule is functionally active (Figure 1A). The binding of bivalent antibodies and their monovalent Fab fragments to cells also differs by approximately 10-fold in off rate (Mason and Williams, 1986). We also analyzed the binding to the 2B4 TCR of IE^k containing the partial agonist peptide, MCC(102S). This peptide contains a Thr to Ser substitution at a TCR contact site that reduces its activity approximately 100-fold in in vitro T cell activation assays (Matsui et al., 1994). As shown in Figure 1B, the monomer dissociates from the 2B4 TCR about 7-fold faster than MCC/IE^k (Figure 1B) (Matsui et al., 1994; Lyons et al., 1996). Likewise, the MCC(102S)/IE^k tetramer has a half-life approximately 13-fold shorter than the MCC/IE^k tetramer, more closely resembling the MCC/IE^k dimer (Figure 1B).

By virtue of their dual specificity, the oligomeric MHC complexes used above contain different valencies of both TCR and coreceptor binding sites. To control for possible CD4 binding effects during later T cell activation studies, we also made a null peptide[MCC(99A)]/IE^k complex and used it to "fill in" unoccupied binding sites on the streptavidin molecules. MCC(99A) is completely inactive in T cell activation assays (Lyons et al., 1996) and when complexed with IE^k has an affinity for

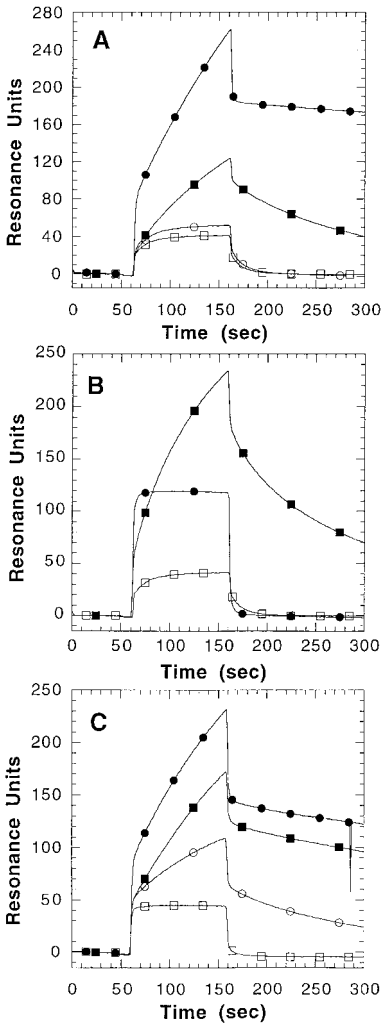

Figure 1. BIAcore Binding Analysis of IE^k Oligomers to the 2B4 TCR

(A) MCC/IE^k complexes were passed over 2B4 TCR coupled to the sensor chip. Shown is the binding of MCC/IE^k-only (open squares), MCC/IE^k monomer (open circles), dimer (solid squares) at 33 μg/ml, and tetramer (solid circles) at 72 μg/ml.

(B) Shown is the binding of MCC/IE^k-only (33 μg/ml) (open squares), MCC(102S)/IE^k (200 μg/ml) (solid circles), and MCC(102S)/IE^k tetramer (100 μg/ml) (solid squares).

(C) Shown is the binding of the mixed oligomers containing MCC(99A)/IE^k in the residual biotin binding sites. Binding was performed at 72 μg/ml monomer (open squares), dimer (open circles), trimer (solid squares), and tetramer (solid circles). Dissociation rates appear faster in (C) because the TCR surface is only half as dense as in (A) and (B). In (A) and (B), concentrations refer to the amount of MCC/IE^k but in (C) they refer to the amount of total IE^k [MCC and MCC(99A)]. No binding was observed with streptavidin only.

the 2B4 TCR too low to measure. Here, the nomenclature refers to the number of MCC/IE^k molecules ("monomer," for example, refers to one MCC/IE^k and three MCC(99A)/IE^k). The binding of monomers and dimers prepared this way is very similar to those without MCC(99A), indicating that even when tightly associated with an active peptide/MHC complex, MCC(99A)/IE^k contributes very little TCR binding energy (Figure 1C). As expected, the trimer dissociates at a rate intermediate between MCC/IE^k dimers and tetramer (Figure 1C). No binding was observed with tetramers of either MCC(99A)/IE^k or MCC(102G)/IE^k at concentrations up to 450 μg/ml (data not shown). MCC(102G) antagonizes the MCC response of the 2B4 hybridoma and when complexed with IE^k binds the 2B4 TCR with an affinity approximately 30-fold lower than MCC (Lyons et al., 1996). A summary of BIAcore-derived half-lives is given in Table 1.

Table 1. Representative $t_{1/2}$ Values for the Binding of MCC/IE^k Complexes to 2B4 TCR

	$t_{1/2}$ Values at 25°C	
Analyte	Low-Density TCR	High-Density TCR
MCC/IE^k	12.0 sec	11.0 sec
MCC/IE^k monomer		9.5 sec
MCC/IE^k dimer	1.7 min	3.1 min
MCC/IE^k tetramer	13.9 min	32.0 min[a]
MCC(102S)/IEk		1.5 sec[b]
MCC(102S)/IE^k tetramer		2.4 min
MCC/IE^k monomer[c]	10.0 sec	14.0 sec
MCC/IE^k dimer[c]	1.9 min	3.4 min
MCC/IE^k trimer[c]	8.3 min	17.0 min

[a] MCC/IEk tetramer off rates show the most variability, possibly due to a greater dependence on TCR density, a higher probability of rebinding, and apparent perturbations in the properties of the hydrogel dextran matrix caused by extensive cross-linking. The latter, which is observed occasionally, is evidence by a nearly flat dissociation phase for the first 2 to 3 min of dissociation followed by more rapid dissociation. Half-lives determined for such curves can be as long as 120 min.

[b] Data taken from Lyons et al. (1996).

[c] These complexes refer to MCC(99A)/IE^k mixed oligomers.

Flow cells contained 3,000–4,000 and 8,000 RU of bound TCR for low- and high-density surfaces, respectively.

Cytosensor Microphysiometer Analysis of T Cell Activation

To have as rapid a readout of T cell activation as possible, we first followed extracellular acidification of T cells using the Cytosensor microphysiometer (McConnell et al., 1992), which detects cellular activation within minutes of stimulation and which has been used to characterize MCC-specific T cells, such as 5C.C7 (Beeson et al., 1996; Rabinowitz et al., 1996b). The 5C.C7 and 2B4 TCRs both use Vα11 and Vβ3 gene segments (Chien et al., 1984; Becker et al., 1985; Fink et al., 1986) and show similarly fine antigen specificity (Hedrick et al., 1982; Reay et al., 1994). The MCC/IE^k tetramer is a potent stimulator of 5C.C7 T cells, giving half-maximal response at 0.1 μg/ml (Figure 2A). Detectable T cell activation occurred with as little as 10 ng/ml tetramer (data not shown). Stimulation by the MCC/IE^k dimer is barely detectable at 2 μg/ml and is still less effective at 20 μg/ml than 0.1 μg/ml tetramer (Figure 2A). We cannot exclude the possibility that small amounts of contaminating tetramer and trimer account for at least some of the activity of dimer (see Experimental Procedures). MCC/IE^k monomer was inactive at 20 μg/ml (Figure 2A), and in two separate experiments no detectable signal occurred with MCC/IE^k-only at a concentration of 200 μg/ml (data not shown). The tetramer containing the partial agonist peptide MCC(102S) stimulates the 5C.C7 T cells weakly at a concentration of 20 μg/ml, while the

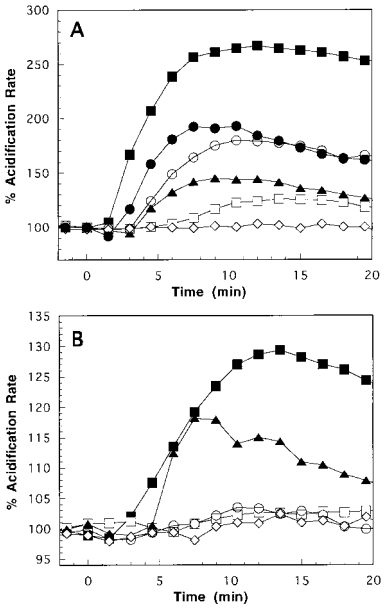

Figure 2. Cytosensor Analysis of T Cell Activation by MCC/IE^k Oligomers

(A) Shown is the 5C.C7 T cell response to MCC/IE^k monomer at 20 μg/ml (open diamonds), MCC/IE^k dimer at 2 μg/ml (open squares) and 20 μg/ml (closed triangle), and MCC/IE^k tetramer at 0.1 μg/ml (open circles), 0.5 μg/ml (closed circles), and 2 μg/ml (closed squares). (B) Shown is the 5C.C7 response to MCC(102G)/IE^k tetramer (open diamonds), MCC(102S)/IE^k tetramer (closed triangles), and MCC/IE^k tetramer (closed squares), at 20, 20, and 0.5 μg/ml, respectively. Also shown is the response to MCC(102S)/IE^k tetramer (open squares) and MCC/IE^k tetramer (open circles) at 20 and 0.5 μg/ml, respectively, following pretreatment of cells with 20 μg/ml GK1.5 antibody. Oligomer concentrations refer to the amount of MCC/IE^k. Maximal response of 5C.C7 cells was approximately 300% using anti-CD3ε antibody (clone 145–2C11), which should oligomerize TCR (Punt et al., 1994).

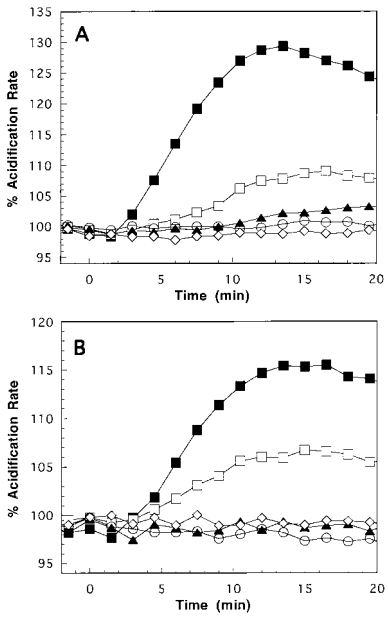

Figure 3. Cytosensor Analysis of T Cell Activation by Mixed Oligomers

(A) Shown is the 5C.C7 T cell response to the mixed oligomers containing both MCC and MCC(99A) for monomer (open circles), dimer (closed triangles), trimer (open squares), and tetramer (closed squares) at 0.5 μg/ml. Also shown is the response to 20 μg/ml MCC(99A)/IE^k tetramer (open diamonds).

(B) Shown is the 2B4 T cell response using same complexes and symbol codes as in (A) except that the concentrations were 1 μg/ml for all samples except MCC(99A)/IE^k tetramer, which was at 20 μg/ml. Concentrations refer to the amount of MCC/IE^k.

tetramer containing the MCC(102G) peptide-antagonist was inactive at this concentration (Figure 2B). Together, these last results indicate that specificity is maintained in this system.

To determine whether this stimulation is CD4 dependent, 5C.C7 T cells were incubated with tetramers of MCC/IE^k or MCC(102S)/IE^k with or without pretreatment with an anti-CD4 antibody (clone GK1.5). GK1.5 completely blocks the response to 0.5 μg/ml of MCC/IE^k tetramer (Figure 2B) and reduces the signal arising from 50 μg/ml of MCC/IE^k tetramer by 60%–70% (data not shown), indicating that CD4 is involved. This is consistent with the lack of T cell activation that occurs by antibody-induced cross-linking of TCR in cells deficient in p56*lck* (Molina et al., 1992; Straus and Weiss, 1992). Likewise, the response to MCC(102S)/IE^k tetramer is inhibited with GK1.5 by more than 90%, indicating that its relatively poor activity is not due to lack of CD4 recruitment (Figure 2B).

In Figure 3A, the response of 5C.C7 T cells to the mixed oligomers containing MCC and MCC(99A) is shown. As is true without MCC(99A) substitution, monomer and dimer are inactive or very weakly active, respectively, at the concentrations tested (Figure 3A). The trimer, although exhibiting a half-life that is only 2-fold less than tetramer in the binding assay, is also markedly less active (Figure 3A). No activation was observed with MCC(99A)/IE^k tetramer at 10 μg/ml (Figure 3A). Experiments with these complexes were also performed with a 2B4 T cell line with similar results (Figure 3B). The fact that the MCC(99A)/IE^k complex could not substitute for MCC/IE^k indicates that CD4 binding alone is not sufficient for T cell stimulation.

Calcium Flux Measurements

To further characterize the T cell signal delivered by tetrameric MCC/IE^k, calcium flux was measured at the single cell level for both 5C.C7 and 2B4 T cells (Figure 4; Table 2). While a commitment to full activation is characterized by a high and sustained level of calcium (Wülfing et al., 1997), only transient elevations are observed in T cells activated with tetramer at concentrations as high as 250 μg/ml (Figure 4; Table 2). Activation above background is seen at tetramer concentrations as low as 100 ng/ml (Table 2). As a further control for specificity, we also found that a hemoglobin (Hb)-specific T cell line was activated by Hb-peptide/IE^k tetramer but not MCC/IE^k tetramer (Table 2). Overall, we conclude that while the MCC/IE^k tetramer is sufficient to initiate signaling, further TCR cross-linking may be necessary for sustained full signals. This conclusion is supported by the ability of MCC/IE^k complexes on planar membranes to induce a sustained calcium flux with these

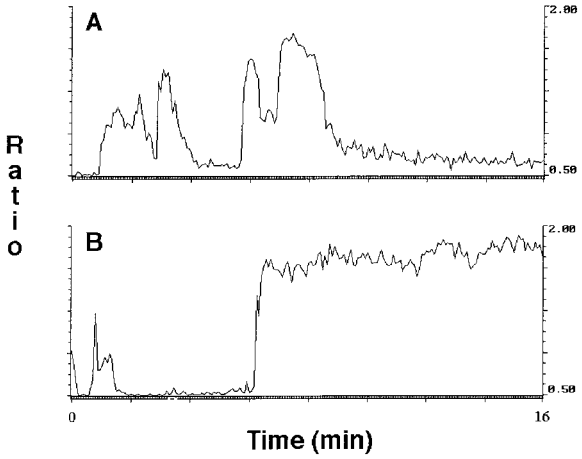

Figure 4. Single Cell Calcium Flux of 5CC7 T Cells

(A) Shown is a representative trace of calcium fluctuations in a 5C.C7 T cell at 37°C treated with MCC/IE^k tetramer at a concentration of 250 μg/ml.

(B) Shown for comparison is a typical calcium response to stimulation by MCC peptide presented on CHO cells transfected with IE^k at 25°C as described in Wülfing et al. (1997). At 25°C, the response to tetramer was generally more lagged than that shown at 37°C.

Table 2. Summary of Calcium Flux Data

	Cells Responding (%) and Type of Calcium Signal			
Stimulus	Cell	Full	Partial	None
MCC/APC	5CC7[a]	90–95	0	5–10
MCC(102S)/APC	5CC7[a]	80–95	5–20	5–10
MCC/IE^k tetramer				
250 μg/ml	5CC7	0	77	23
10–100 μg/ml	2B4	0.8	61	39
0.1 μg/ml	2B4	0	18.5	81.5
Hb/APC	PL-17[b]	50–85	0	15–50
MCC/IE^k tetramer	PL-17	0	12	88
Hb/IEk tetramer	PL-17	0	50	50

[a] Taken from Wülfing et al. (1997). The range in percentages occurs for peptide concentrations of 25 nM to 25 μM.

[b] PL-17 is a T cell line that is specific for a hemoglobin peptide presented by IE^k (Evavold et al., 1992). The range in percentages occurs for peptide concentrations of 100 nM to 100 μM in a single experiment.

The percentage of stimulation by MCC/IE^k tetramer at 250 μg/ml was for a single experiment, at 10–100 μg/ml is an average of four experiments, and at 0.1 μg/ml is an average of two experiments. Background level of calcium signals was always 10% or less.

same T cells (Wülfing et al., 1998). In an APC-T cell conjugate, TCRs and signaling elements can concentrate within contact patches, while tetramer-ligated TCRs should at least initially be uniformly distributed, possibly diluting limiting amounts of an initiation kinase, as has been shown to occur for the IgE receptor (Torigoe et al., 1997).

Discussion

The data presented here are not consistent with a conformational model of signaling through individually engaged TCRs. This is because monomeric MCC/IE^k fails to activate T cells at a concentration 20,000 times higher than stimulatory doses of MCC/IE^k tetramer, despite the fact that occupancy of TCR by MCC/IE^k should be much higher under these conditions (see Experimental Procedures and Table 1). These arguments exclude occupancy, as opposed to clustering, as a reason for the much greater potency of tetramer versus monomer and dimer. The presence of the CD4 coreceptor on T cells should not significantly alter these occupancy estimates, since the GK1.5 antibody only marginally inhibits FACS staining of 5C.C7 and 2B4 T cell lines with MCC/IE^k tetramers (data not shown). A lack of CD4 enhancement of MHC-tetramer binding to T cells has also been recently reported by Crawford et al. (1998). Nevertheless, it is clear from the data shown here that stimulation by MHC tetramers is CD4 dependent. This discrepancy between CD4's role in activation versus binding is consistent with evidence that coreceptors may interact by a sequential mechanism with preformed Ag/MHC-TCR complexes (Hampl et al., 1997) and possibly in a substoichiometric manner (Garcia et al., 1996). Additionally, Abastado et al. (1995) has also shown that monomeric class I MHC-peptide complexes were inactive at stimulating T cells. To date, available crystallographic data on Ag/MHC-TCR complexes show no evidence of global structural changes in the TCR upon binding (Garboczi et al., 1996; Garcia et al., 1998).

Because biotin binding sites in streptavidin are arranged in a tetrahedral configuration (Pahler et al., 1987), three of four MCC/IE^k molecules should form one face of a tetrahedron and contact the T cell surface (as modeled by McMichael and O'Callaghan, 1998). Thus, the ability to stabilize the colocalization of two or three TCRs can explain the tetramers striking potency. Nevertheless, the possibility that activation occurs because TCRs are ligated for longer periods of time, as opposed to clustering per se, cannot be formally excluded. A dimerization model of signaling has been widely discussed as a possible early trigger of T cell activation since it was first proposed by Brown et al. (1993) based on their observation that the class II MHC molecule, DR1, crystallized as a dimer of the heterodimer with a symmetry that could allow simultaneous engagement of two TCRs on a cell surface. A similar observation has been made with the class II molecule, DR3 (Ghosh et al., 1995). In contrast, an IE^k crystal structure had a dimer possessing a symmetry inconsistent with coligation of two TCRs (Fremont et al., 1996), and several other class II crystal structures have lacked dimers (Dessen et al., 1997; Fremont et al., 1998; Scott et al., 1998). Abastado et al. (1995) used an antibody to create dimers of a class I MHC-peptide complex and found that these stimulate IL-2 production in a T cell hybridoma and seratonin release from a basophil transfectant. However, they also showed that higher-order aggregates in such preparations contained more activity than isolated dimers (Abastado et al., 1995). Although not titrated, the concentration of size-fractionated dimers used to activate T cells was apparently far greater than those concentrations reported here for tetramers (Abastado et al., 1995). Any intrinsic stimulatory activity in our dimers seems to be present at levels too low to be physiologically relevant. In contrast to our results, Abastado et al. (1995)

report that dimers induce full signals. This discrepancy may be explained by their longer assays, which could allow Ag/MHC-TCR dimers to further aggregate by diffusion on cells into larger clusters. Alternatively, the streptavidin backbone used here may create a less optimal alignment of TCRs than the antibody approach.

The large increase in activity of tetramer versus dimer suggests an interesting alternative to a dimerization-based mechanism: that the colocalization of at least three TCRs is required to form a critical level of signaling, as seems to occur for the TNF receptor (Banner et al., 1993). The significantly diminished activity of the MCC/IE^k trimer, relative to tetramer, is also consistent with TCR trimerization as a critical event, since all possible combinations of three MCC/IE^k complexes are active on the tetramer compared to only one on the trimer.

Unlike growth-factors that contain two receptor binding sites per molecule, a single TCR engages only one Ag-MHC complex at a time; therefore, there is no reason to suspect that clustering of TCRs should end with dimers or trimers. A gradient toward more complete activation could occur with the formation of larger clusters, as suggested here by the calcium flux experiments. Support for such a model comes from work by Dintzis and colleagues who used defined polymers of hapten to stimulate hapten-specific T cells (Symer et al., 1992). They conclude that a minimal number of antigen receptors must be cross-linked to form an "Immunon," or essential immunogenic signaling structure (Dintzis et al., 1989; Symer et al., 1992). More recently, Rotzschke et al. (1997), using polypeptide oligomers of peptide antigens to cross-link MHC molecules on the cell surface, showed that higher order oligomers gave better T cell activation, reaching a plateau at about 16 antigenic peptide units. More directly, Reich et al. (1997) recently showed that productive Ag-MHC/TCR complexes can self-associate in solution to form supramolecular structures containing six or more primary complexes. Thus, the Ag-MHC/TCR complex has an affinity for itself that neither TCR nor MHC alone possesses, making the half-life of the antigen-specific binding event a determinant of the degree of clustering. The puzzle of how specific Ag/MHC-TCR complexes accumulate in a cluster in the presence of a large excess of irrelevant antigen could then be explained by their longer half-lives (Reich et al., 1997). The appeal of such a model is that the sensitivity and range of T cell responses to antigen can be explained largely by kinetics and the resulting differences in the degree of clustering. An example shown here is that a partial agonist tetramer containing only a 7-fold-weaker monomeric interaction [MCC(102S) versus MCC] is reduced in activity approximately 500-fold relative to wild-type tetramer, presumably because of a diminished ability to kinetically drive TCR clustering. The serial engagement of many TCRs by a few Ag/MHC complexes may also explain the high antigen sensitivity of T cells (Valitutti and Lanzavecchia, 1997).

What is the advantage of signaling by a receptor clustering mechanism? The colocalization of TCRs may allow sufficient cross-phosphorylation to occur, a reaction that is thought to be very dependent on steric constraints and local substrate concentrations (reviewed in Ortega, 1995) and may be of particular importance for those receptors that lack intrinsic kinase activity. Unlike most receptors that are paired with a specific ligand and respond only to this ligand, antigen receptors must distinguish between a large number (>2000) of self and non-self ligands of very similar chemical composition. Indeed, other antigen receptors on B lymphocytes (surface immunoglobulin and Fc receptor) may also transmit signals to the inside of the cell by a clustering mechanism (reviewed in Alberola-Ila et al., 1997; Reth and Wienands, 1997; Daeron, 1997). Clustering, with its inherent time lag, may filter out the transient irrelevant interactions that occur when a T cell scans another cell during immune surveillance. As previously suggested (McKeithan, 1995; Rabinowitz et al., 1996a), the separation of binding and signaling by several steps (oligomerization included) can explain the TCR's ability to finely discriminate, both quantitatively and qualitatively, between chemically similar antigens that differ only modestly in their binding affinities.

Experimental Procedures

Synthetic Peptides

Synthetic peptides were prepared by the Stanford Protein and Nucleic Acid Facility by FMOC chemistry and confirmed by amino acid analysis and mass spectrometry. MCC peptide (residues 88–103) was ANERADLIAYL**K**QA**T**K. MCC(99A) contained an Ala instead of the highlighted Lys residue. MCC(102S) and MCC(102G) contained a Ser and Gly, respectively, instead of the highlighted Thr residue. Hb (β^{dmin} residues 53–76) peptide had the sequence AIMGNPKVKAH GKKVITAFNEGLK.

Proteins

Recombinant IE^k containing a single C-terminal 15 amino acid biotinylation site was produced as described elsewhere (Altman et al., 1993, 1996) with the following changes. Inclusion bodies containing IE^k chains were prepared from the BL21(DE3)pLysS strain of *Escherichia coli*, by repeated freeze/thaw/wash cycles in 50 mM Tris (pH 8.0), 1 mM EDTA, 25% sucrose, 1% Triton X-100, and 1 mg/ml Lysozyme. The inclusion body preparation was then dissolved in 5.8 M guanidine-HCl, 50 mM Tris (pH 8.8), and 2 mM EDTA. Chains were allowed to oxidize individually to form disulfide bonds for 5–7 days at less than 1 mg/ml in this buffer. Following concentration, folding was initiated by combining and diluting subunits to 2 μM in 50 mM sodium phosphate (pH 7.5), 25% glycerol, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 0.5 mM EDTA, 0.1 mM PMSF, 1 μg/ml pepstatin and leupeptin, and 10 μM antigenic peptide. After 4–5 days of incubation at 4°C, correctly folded peptide/IE^k complexes were isolated by immunoaffinity chromatography on a 14.4.4 MAb column. Ag/IE^k complexes were then enzymatically biotinylated as described (Altman et al., 1996) except that protease inhibitors were included and the protein was later size purified to remove aggregates and free biotin. Biotinylation was confirmed by a gel-shift assay using excess streptavidin and 10% SDS-PAGE gels under nondenaturing conditions and was typically greater than 75%.

Preparation of Oligomers

An FPLC Superdex-200 column (10/30) was used for all gel filtration steps below. Streptavidin was purchased from Prozyme, Inc. (San Leandro, CA). In initial studies, stimulatory activity was found in MCC/IE^k-only prior to gel filtration, indicating that great care had to be taken to remove aggregates. Given their relative potencies, it is clear that less than 0.01% contamination of a tetrameric aggregate in an MCC/IE^k preparation can account for significant activity. Therefore, streptavidin and Ag/IE^k-biotin were size purified before reacting, after which oligomers were size purified again just before use. Size purification of MCC/IE^k tetramer immediately prior to experiments and without subsequent reconcentration did not remove its stimulatory activity. The 55 kDa form of streptavidin adsorbs strongly to the FPLC column in PBS and eluted late. Tetramers were

prepared by adding streptavidin in several steps to a 20%–40% molar excess of Ag/IE^k-biotin over biotin binding sites and isolating by gel filtration. SA-linked monomers and dimers were produced at approximately a 60/40 ratio by a single reaction using a 3- to 4-fold molar excess of streptavidin over MCC/IE^k-biotin (12- to 16-fold excess of biotin binding sites). MCC/IE^k dimer required one additional gel filtration step before use but probably contained small amounts of contaminating trimer and tetramer because in one experiment a third gel filtration step significantly reduced its T cell stimulatory activity. Tetramers and dimers eluted as expected for their size, indicating that the attached IE^k was sufficient to correct the adsorbing tendency of streptavidin. Streptavidin-linked monomer showed some residual adsorbing behavior, eluting slightly later than IE^k, and required no further purification. The composition of these oligomers was confirmed by SDS-PAGE and by BIAcore binding kinetics. For MCC/99A mixed oligomers, monomer was formed by first making MCC/IE^k monomer as described above and then reacting with excess MCC(99A)/IE^k-biotin. Dimer and trimer were prepared by first making MCC(99A)/IE^k monomer and dimer as described for MCC and then reacting with MCC/IE^k-biotin. These mixed tetramers were then purified by gel filtration. Protein concentrations were determined by absorbance at 280 nm using extinction coefficients of 1.3 ml mg^{-1} cm^{-1} for IE^k and 3.2 ml mg^{-1} cm^{-1} for streptavidin.

T Cells

The 5C.C7 and 2B4 T cell lines were prepared from the spleen and lymph nodes, respectively, of 5C.C7 and 2B4 TCR transgenic mice and were maintained in culture by in vitro priming regularly with irradiated splenocytes and antigen. The primary 2B4 T cells used for calcium flux were prepared from lymph nodes of 2B4 TCR transgenic mice and were used on day 8–12 after a single in vitro priming with irradiated splenocytes and antigen.

BIAcore Analysis

Soluble 2B4 TCR was immobilized on the BIAcore CM5 chip by standard PDEA-mediated thiol coupling. Oriented coupling occurs via a single free thiol in the C-beta domain. Analyte binding was monitored at a flow rate of 15 μl/min and 25°C. Dissociation phases were fit using the Marquardt-Levenberg nonlinear least squares algorithm provided in the BIAEvaluation 2.1 package using a simple Langmuir dissociation model. The apparent dissociation rate constants were used to determine the half-lives. These are used only to illustrate the relative differences in avidity and do not represent accurate rate constants since the simple Langmuir model is not appropriate for multivalent interactions. Standard errors were typically less than 3% of the value of the fitted rate constant.

Unlike a 2-dimensional cell surface, the BIAcore hydrogel-matrix contains a 3-dimensional TCR array supported by linear dextran molecules with some diffusional flexibility. Calculations show that 8,000 RU of immobilized TCR fills 12%–25% of the available volume of the matrix (neglecting the dextran molecules), and MCC/IE^k tetramers are about four times larger than TCR molecules. Under these conditions, it seems likely that many tetramers may engage four TCRs simultaneously, indicating that the BIAcore off rate should reflect an upper limit to tetramer avidity. Thus, from the half-lives in Table 1, the avidity of the MCC/IE^k tetramer can be estimated to be 170 times greater than MCC/IE^k-only. This is probably an overestimate of the avidity of the tetramer on T cells where all four MHC molecules may not be able to simultaneously engage TCRs (see Discussion).

Cytosensor Microphysiometer Analysis

Acid release was measured similarly to that described previously (Beeson et al., 1996; Rabinowitz et al., 1996b), except that T cells-only (5C.C7 or 2B4 T cell lines) were loaded in the cell chamber without APCs. Baseline acid release rates were 30–100 μV sec^{-1}. IE^k samples were diluted into low-buffer RPMI before injection into the cell chamber, and when necessary IE^k stocks were exchanged into low-buffer (1 mM sodium phosphate) phosphate-buffered saline during gel filtration.

Calcium Imaging

The 5C.C7 T cell line and the primary 2B4 lymphocytes were calcium imaged similarly to that described elsewhere (Wülfing et al., 1997). CHO cells, either untransfected or transfected with native IE^k as a positive control, were grown to confluency on cover slips (4.8 × 6.0 cm). Cover slips with CHO-IE^k were pulsed overnight with MCC peptide at 25 μM. Cover slips were then joined to a custom made flow chamber to form the bottom of the imaged well (height, 0.26 mm; width, 5 mm; length, 40 mm). Fura-2-loaded T cells (15 μl), either alone or immediately after mixing 1:1 with IE^k oligomer, were injected into the cell chamber by aspiration. T cells that settled onto the CHO focal plane were analyzed individually for intracellular calcium levels as described (Wülfing et al., 1997). In Table 2, full signals refer to elevations in intracellular calcium that are sustained for several minutes and typically involve a single trigger point. Partial signals may or may not be repetitive in nature but are of relatively short duration and returns to baseline (Wülfing et al., 1997). The lag time between mixing of MHC samples and T cells and the start of data collection was typically less than 30 sec.

Acknowledgments

We thank Loan Nguyen, Suzanne Erickson-Ybarra, Sharmila Sudanagunta, Dan Lyons, Brian Trenchak, and Kristin Baldwin for their help in the preparation of IE^k and 2B4, Korey Singleton for early dimer studies, Ashley Chi for RT-PCR help, Paul Allen for PL17, Cathy Carswell-Crumpton for cell sorting assistance, Susan Palmieri and Mike Sjaastad for help with calcium imaging, Max Krummel for discussions, and Peter Shatz for early supplies of the BirA enzyme. J. J. B. was supported by a National Institutes of Health training grant and by a fellowship from the Irvington Institute for Medical Research, J. D. R. by the Medical Scientist Training Program, C. W. by a European Molecular Biology Organization long-term fellowship and the Howard Hughes Medical Institute, C. B. by a fellowship from the Cancer Research Institute, and J. D. A. by a fellowship from the American Cancer Society. This work was supported by grants from the National Institutes of Health (H. M. M. and M. M. D.) and the Howard Hughes Medical Institute (M. M. D.).

Received June 5, 1998; revised August 17, 1998.

References

Abastado, J-P., Lone, Y-C., Casrouge, A., Boulot, G., and Kourilsky, P. (1995). Dimerization of soluble major histocompatibility complex-peptide complexes is sufficient for activation of T cell hybridoma and induction of unresponsiveness. J. Exp. Med. *182*, 439–447.

Alberola-Ila, J., Satoshi, T., Kerner, J.D., and Perlmutter, R.M. (1997). Differential signaling by lymphocyte antigen receptors. Annu. Rev. Immunol. *15*, 125–154.

Altman, J.D., Reay, P.A., and Davis, M.M. (1993). Formation of functional peptide complexes of class II major histocompatibility complex proteins from subunits produced in Escherichia coli. Proc. Natl. Acad. Sci. USA *90*, 10330–10334.

Altman, J.D., Moss, P.A.H., Goulder, P.J.R., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94–96.

Banner, D.W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H-J., Broger, C., Loetscher, H., and Lesslauer, W. (1993). Crystal structure of the soluble human 55 kd TNF receptor-human TNFβ complex: implications for TNF receptor activation. Cell *73*, 431–445.

Beeson, C., Rabinowitz, J.D., Tate, K., Gütgemann, I., Chien, Y.H., Jones, P.P., Davis, M.M., and McConnell, H.M. (1996). Early biochemical signals arise from low affinity TCR-ligand reactions at the cell-cell surface. J. Exp. Med. *184*, 777–782.

Becker, D.M., Patten, P., Chien, Y., Yokota, T., Eshhar, Z., Giedlin, M., Gascoigne, N.R., Goodnow, C., Wolf, R., Arai, K., et al. (1985). Variability and repertoire size of T-cell receptor V alpha gene segments. Nature *317*, 430–434.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G.,

Strominger, J.L., and Wiley, D.C. (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1, Nature *364*, 33–39.

Chien, Y.H., Gascoigne, N.R., Kavaler, J., Lee, N.E., and Davis, M.M. (1984). Somatic recombination in a murine T-cell receptor gene. Nature *309*, 322–326.

Crawford, F., Kozono, H., White, J., Marrack, P., and Kappler, J. (1998). Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. Immunity *8*, 675–682.

Daeron, M. (1997). Fc receptor biology. Annu. Rev. Immunol. *15*, 203–234.

Davis, M.M., Boniface, J.J., Reich, Z., Lyons, D.S., Hampl, J., Arden, B., and Chien, Y.-h. (1998). Ligand recognition by $\alpha\beta$ T cell receptors. Annu. Rev. Immunol. *16*, 523–544.

Dessen, A., Lawrence, C.M., Cupo, S., Zaller, D.M., and Wiley, D.C. (1997). X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. Immunity *7*, 473–481.

Dintzis, R.Z., Okajima, M., Middleton, M.H., Green, G., and Dintzis, H.M. (1989). The immunogenicity of soluble haptenated polymers is determined by molecular mass and hapten valence. J. Immunol. *143*, 1239–1244.

Evavold, B.D., Williams, S.G., Hsu, B.L., Buus, S., and Allen, P.M. (1992). Complete dissection of the Hb(64-76) determinant using T helper 1, T helper 2 clones, and T cell hybridomas. J. Immunol. *148*, 347–353.

Fink, P.J., Matis, L.A., McElligott, D.L., Bookman, M., and Hedrick, S.M. (1986). Correlations between T-cell specificity and the structure of the antigen receptor. Nature *321*, 219–226.

Fremont, D.H., Hendrickson, W.A., Marrack, P., and Kappler, J.W. (1996). Structures of an MHC class II molecule with covalently bound single peptides. Science *272*, 1001–1004.

Fremont, D.H., Monnaie, D., Nelson, C.A., Hendrickson, W.A., and Unanue, E.R. (1998). Crystal structure of I-A^k in complex with a dominant epitope of lysozyme. Immunity *8*, 305–317.

Garboczi, D.N., Ghosh, P., Utz, U., Fan, Q.R., Biddison, W.E., and Wiley, D.C. (1996). Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. Nature *384*, 134–141.

Garcia, K.C., Scott, C.A., Brunmark, A., Carbone, F.R., Peterson, P.A., Wilson, I.A., and Teyton, L. (1996). CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes. Nature *384*, 577–581.

Garcia, K.C., Degano, M., Pease, L.R., Huang, M., Peterson, P.A., Teyton, L., and Wilson, I.A. (1998). Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. Science *279*, 1166–1172.

Ghosh, P., Amaya, M., Mellins, E., and Wiley, D.C. (1995). The structure of an intermediate in class II MHC maturation: clip bound to HLA-DR3. Nature *378*, 457–462.

Gütgemann, I., Fahrer, A.M., Altman, J.D., Davis, M.M., and Chien, Y-h. (1998). Induction of rapid T cell activation and tolerance by systemic presentation of an orally administered antigen. Immunity *8*, 667–673.

Hampl, J., Chien, Y-h., and Davis, M.M. (1997). CD4 augments the response of a T cell to agonist but not to antagonist ligands. Immunity *7*, 379–385.

Hedrick, S.M., Matis, L.A., Hecht, T.T., Samelson, L.E., Longo, D.L., Heber-Katz, E., and Schwartz, R.H. (1982). The fine specificity of antigen and Ia determinant recognition by T cell hybridoma clones specific for pigeon cytochrome c. Cell *30*, 141–152.

Kappler, J., Kubo, R., Haskins, K., White, J., and Marrack, P. (1983). The mouse T cell receptor: comparison of MHC-restricted receptors in two T cell hybridomas. Cell *34*, 727–737.

Karjalainen, K. (1994). High sensitivity, low affinity-paradox of T-cell receptor recognition. Curr. Opin. Immunol. *6*, 9–12.

Kaye, J., Porcelli, S., Tite, J., Jones, B., and Janeway, C.A., Jr. (1983). Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen presenting cells in activation of T cells. J. Exp. Med. *158*, 836–856.

Kolanus, W., Romeo, C., and Seed, B. (1993). T cell activation by clustered tyrosine kinases. Cell *74*, 171–183.

Lyons, D.S., Lieberman, S.A., Hampl, J., Boniface, J.J., Reay, P.A., Chien, Y., Berg, L.J., and Davis, M.M. (1996). T cell receptor binding to antagonist peptide/MHC complexes exhibits lower affinities and faster dissociation rates than to agonist ligands. Immunity *5*, 53–61.

Mason, D.W., and Williams, A.F. (1986). Kinetics of antibody reactions and the analysis of cell surface antigens. In Handbook of Experimental Immunology, D.M. Weir et al., eds. (Oxford: Blackwell Scientific Publications), Volume 1, Chapter 38.

Matsui, K., Boniface, J.J., Steffner, P., Reay, P.A., and Davis, M.M. (1994). Kinetics of T-cell receptor binding to peptide/I-E^k complexes: correlation of the dissociation rate with T-cell responsiveness. Proc. Natl. Acad. Sci. USA *91*, 12862–12866.

McConnell, H.M., Owicki, J.C., Parce, J.W., Miller, D.L., Baxter, G.T., Wada, H.G., and Pitchford, S. (1992). The cytosensor microphysiometer: biological applications of silicon technology. Science *257*, 1906–1912.

McKeithan, T.W. (1995). Kinetic proofreading in T-cell receptor signal transduction. Proc. Natl. Acad. Sci. USA *92*, 5042–5046.

McMichael, A.J., and O'Callaghan, C.A. (1998). A new look at T cells. J. Exp. Med *187*, 1367–1371.

Meuer, S.C., Hodgdon, J.C., Hussey, R.E., Protentis, J.P., Schlossman, S.F., and Reinherz, E.L. (1983). Antigen-like effects of monoclonal antibodies directed at receptors on human T cell clones. J. Exp. Med. *158*, 988–995.

Molina, T.J., Kishihara, K., Siderovski, D.P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C.J., Hartmann, K.U., Veillette, A., et al. (1992). Profound block in thymocyte development in mice lacking p56*lck*. Nature *357*, 161–164.

Ortega, E. (1995). How do multichain immune recognition receptors signal? A structural hypothesis. Mol. Immunol. *32*, 941–945.

Pahler, A., Hendrickson, W.A., Kolks, M.A., Argarana, C.E., and Cantor, C.R. (1987). Characterization and crystallization of core streptavidin. J. Biol. Chem. *226*, 13933–13937.

Punt, J.A., Roberts, J.L., Kearse, K.P., and Singer, A. (1994). Stoichiometry of the T cell antigen receptor (TCR) complex: each TCR/CD3 complex contains one TCRα, one TCRβ, and two CD3ϵ chains. J. Exp. Med. *180*, 587–593.

Rabinowitz, J.D., Beeson, C., Lyons, D.S., Davis, M.M., and McConnell, H.M. (1996a). Kinetic discrimination in T-cell activation, Proc. Natl. Acad. Sci. USA *93*, 1401–1405.

Rabinowitz, J.D., Beeson, C., Wülfing, C., Tate, K., Allen, P.M., Davis, M.M., and McConnell, H.M. (1996b). Altered T cell receptor ligands trigger a subset of early T cell signals. Immunity *5*, 125–135.

Reay, P.A., Kantor, R.M., and Davis, M.M. (1994). Use of global amino acid replacement to define the requirements for MHC binding and T cell recognition of moth cytochrome c (93–103). J. Immunol. *152*, 3946–3957.

Reich, Z., Boniface, J.J., Lyons, D.S., Borochov, N., Wachtel, E.J., and Davis, M.M. (1997). Ligand-specific oligomerization of T-cell receptor molecules. Nature *387*, 617–620.

Reth, M., and Wienands, J. (1997). Initiation and processing of signals from the B cell antigen receptor. Annu. Rev. Immunol. *15*, 453–479.

Rotzschke, O., Falk, K., and Strominger, J.L. (1997). Superactivation of an immune response triggered by oligomerized T cell epitopes. Proc. Natl. Acad. Sci. USA *94*, 14642–14647.

Samelson, L.E., Germain, R.N., and Schwartz, R.H. (1983). Monoclonal antibodies against the antigen receptor on a cloned T-cell hybrid. Proc. Natl. Acad. Sci. USA *22*, 6972–6976.

Scott, C.A., Peterson, P.A., Teyton, L., and Wilson, I.A. (1998). Crystal structures of two I-A^d-peptide complexes reveal that high affinity can be achieved without large anchor residues. Immunity *8*, 319–329.

Shaw, A.S., Chalupny, J., Whitney, J.A., Hammond, C., Amrein, K.E., Kavathas, P., Sefton, B.M., and Rose, J.K. (1990). Short related sequences in the cytoplasmic domains of CD4 and CD8 mediate binding to the amino-terminal domain of the p56^{lck} tyrosine kinase. Mol. Cell. Biol. *10*, 1853–1862.

Spencer, D.M., Wandless, T.J., Schreiber, S.L., and Crabtree, G.R. (1993). Controlling signal transduction with synthetic ligands. Science *262*, 1019–1024.

Straus, D.B., and Weiss, A. (1992). Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. Cell *70*, 585–593.

Symer, D.E., Dintzis, R.Z., Diamond, D.J., and Dintzis, H.M. (1992). Inhibition or activation of human T cell receptor transfectants is controlled by defined, soluble antigen arrays. J. Exp. Med. *176*, 1421–1430.

Takahama, Y., Suzuki, H., Katz, K.S., Grusby, M.J., and Singer, A. (1994). Positive selection of $CD4^+$ T cells by TCR ligation without aggregation even in the absence of MHC. Nature *371*, 67–70.

Torigoe, C., Goldstein, B., Wofsy, C., and Metzger, H. (1997). Shuttling of initiating kinase between discrete aggregates of the high affinity receptor for IgE regulates the cellular response. Proc. Natl. Acad. Sci. USA *94*, 1372–1377.

Turner, J.M., Brodsky, M.H., Irving, B.A., Levin, S.D., Perlmutter, R.M., and Littman, D.R. (1990). Interaction of the unique N-terminal region of the tyrosine kinase p56*lck* with the cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. Cell *60*, 755–765.

Valitutti, S., and Lanzavecchia, A. (1997). Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. Immunol. Today *18*, 299–304.

Watts, T.H. (1988). T cell activation by preformed, long-lived Ia-peptide complexes: quantitative aspects. J. Immunol. *141*, 3708–3714.

Wülfing, C., Rabinowitz, J.D., Beeson, C., Sjaastad, M.D., McConnell, H.M., and Davis, M.M. (1997). Kinetics and extent of T cell activation as measured with the calcium signal. J. Exp. Med. *185*, 1815–1825.

Wülfing, C., Sjaastad, M.D., and Davis, M.M. (1998). 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. Proc. Natl. Acad. Sci. USA *95*, 6302–6307.