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TCR–peptide–MHC interactions *in situ* show accelerated kinetics and increased affinity

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Abstract

The recognition of foreign antigens by T lymphocytes is essential to most adaptive immune responses. It is driven by specific T-cell antigen receptors (TCRs) binding to antigenic peptide–major histocompatibility complex (pMHC) molecules on other cells¹. If productive, these interactions promote the formation of an immunological synapse^{2,3}. Here we show that synaptic TCR–pMHC binding dynamics differ significantly from TCR–pMHC binding in solution. We used single-molecule microscopy and fluorescence resonance energy transfer (FRET) between fluorescently tagged TCRs and their cognate pMHC ligands to measure the kinetics of TCR–pMHC binding *in situ*. When compared with solution measurements, the dissociation of this complex was increased significantly (4–12-fold). Disruption of actin polymers reversed this effect, indicating that cytoskeletal dynamics destabilize this interaction directly or indirectly. Nevertheless, TCR affinity for pMHC was significantly elevated as the result of a large (about 100-fold) increase in the association rate, a likely consequence of complementary molecular orientation and clustering. In helper T cells, the CD4 molecule has been proposed to bind cooperatively with the TCR to the same pMHC complex. However, CD4 blockade had no effect on the synaptic TCR affinity, nor did it destabilize TCR–pMHC complexes, indicating that the TCR binds pMHC independently of CD4.

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Author Contributions J.B.H., G.J.S. and M.M.D. conceived the project. Reagents and experimental systems were designed and tested by J.B.H. unless indicated otherwise. M.A., M.A.M. and G.J.S. provided important expertise in single-molecule microscopy. M.A. conducted all single dye tracing experiments and all smFRET experiments performed in Linz. J.B.H. conducted all bulk FRET experiments and all smFRET experiments at Stanford. J.B.H. and M.A.M. laid the experimental foundation in the initial phase of the project. M.A.M. provided important expertise in setting up the microscope system at Stanford. B.F.L. provided the expression system for ICAM-1 and B7-1. E.W.N. conducted all SPR measurements. M.B. conducted all TOCCSL experiments. L.O.K. and B.F.L. contributed important ideas. J.B.H. and M.M.D. wrote the manuscript.

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Surface plasmon resonance (SPR) and microcalorimetry experiments with engineered soluble proteins generally show weak TCR–pMHC binding, with affinities (K_d values) in the range 1–100 μM , $t_{1/2}$ values on the order of seconds, and association rates usually ranging from 1,000 to 10,000 $\text{M}^{-1} \text{s}^{-1}$ (refs 4–6). Nevertheless, T cells are highly specific and sensitive for antigen, able to detect even a single antigenic pMHC complex among structurally similar yet non-stimulatory pMHCs^{7,8}. However, although this is useful for comparative purposes, these measurements do not account for the many constraints and special features of the synaptic environment and might not reflect what occurs *in situ*. In particular, the restricted intercellular volume should greatly accelerate the association rate and enhance serial engagement^{9–11}. Favourable molecular alignment of TCR and MHC, as well as any molecular pre-clustering, could also drastically affect binding^{12–14}. Although the volume effect can be approximated, the influence of the others is essentially unknown. We have therefore devised a method to measure synaptic TCR binding to pMHC directly, using single-molecule microscopy and FRET between a donor fluorophore on the TCR and an acceptor fluorophore on the peptide bound to a MHC molecule.

We used T-cell blasts from two different TCR transgenic T-cell lines, 2B4 and 5c.c7, which are specific for the same moth cytochrome *c* peptide (MCC 88–103) bound to the class II MHC molecule IE^k. To permit the use of highly sensitive and rapid total internal reflection (TIRF) microscopy, we used a modified planar lipid bilayer system as a surrogate antigen-presenting cell surface, presenting IE^k complexes, ICAM-1 adhesion molecules and B7-1 co-stimulatory polypeptides (see Methods).

Structural analysis of an Fab fragment derived from the monoclonal anti-TCR- β antibody H57 complexed with the C β region of a TCR indicated that portions of its binding site might be close enough to the carboxy terminus of a peptide bound to a MHC (about 41 Å)^{15,16} to allow FRET *in situ* to measure TCR binding. We therefore constructed a single-chain variable fragment (scFv) of this antibody with mutations that could be labelled at three different sites (J1, J2 and J3). Their location and proximity to the C terminus of a peptide bound to the IE^k molecule are shown (Fig. 1a).

On addition of the Cy3-labelled J1 reagent to 5c.c7 transgenic T cells on bilayers containing IE^k/MCC(C)-Cy5, we observed the rapid onset of a FRET signal, which was corrected for donor bleed-through and acceptor cross-excitation (see Methods). Consistent with the FRET results was the observation that this signal disappeared immediately after ablation of the FRET acceptor, together with an increase in donor intensity (Fig. 1b). Reversing the labels produced comparable results (Fig. 1b and Supplementary Fig. 1a). The average synaptic FRET yield (as measured by donor recovery after acceptor bleaching; see Methods) was 10–12% for the agonists IE^k/MCC(C)-Cy5 and IE^k/K5(C)-Cy5 (Fig. 1c). FRET was highly dependent on the ligand: no synaptic FRET was observed between the J1-Cy3-labelled TCR and the null ligands IE^k/ $\beta_2\text{M}$ (C)-Cy5 or IE^k/MCC null(C)-Cy5, even in the presence of unlabelled agonists (IE^k/MCC) to ensure T-cell activation (Fig. 1b, c). The FRET strength correlated with both the density of labelled IE^k/peptide(C)-Cy5 and the ligand potency (Supplementary Fig. 1b). No FRET was detectable when T cells were labelled with a Cy3-conjugated anti-CD4 Fab (GK1.5), further validating signal specificity (Fig. 1c).

To assess potential bystander FRET between TCR–pMHC complex bound J1-Cy3 and neighbouring pMHCs, we tested the J1, J2 and J3 probes in conjunction with the agonist ligand IE^k/K5 labelled at either the C or N terminus of the peptide. These combinations gave rise to six inter-dye distances ranging from about 41 Å to about 100 Å. Average synaptic FRET depended strictly on the distance between the dye pair, as expected, given that yields of FRET decline inversely with the sixth power of the distance (Fig. 1d). Hence, this assay is specific for individual TCR–ligand interactions, and bystander effects are negligible.

We also used single-molecule FRET (smFRET) microscopy to derive dissociation rates. After correcting for donor bleed-through and acceptor cross-excitation, we identified smFRET events as those that both co-localized with single acceptor molecules and also appeared and disappeared in a single step—a hallmark of single-molecule behaviour (Fig. 2a and Supplementary Figs 2–7). Furthermore, the appearance of these events was specific to label and ligand: other label combinations, including J3-Cy3 or IE^k/MCC-null(C)-Cy5, did not produce such events (data not shown). Reversing the label proportion by employing IE^k/MCC(C)-Cy5 in high abundance and J1-Cy3 in low abundance also led to smFRET events (Supplementary Figs 8–10). By monitoring the duration of these FRET signals during synapse formation and correcting for photobleaching (see Methods), we determined the dissociation rates for both the 5c.c7 TCR and the 2B4 TCR with IE^k/MCC at 37 °C and for 5c.c7 with the weak agonist T102S (Fig. 2b, Table 1 and Supplementary Fig. 1c). These data followed single-exponential decay kinetics, which is consistent with a simple dissociation of the TCR–pMHC complex. Unexpectedly, the $t_{1/2}$ values for these interactions were much shorter than those measured *in vitro* by SPR, especially for the 5c.c7 TCR (Fig. 2c). Across a temperature range we found that synaptic $t_{1/2}$ values were consistently 3–12-fold shorter than those measured *in vitro*, especially at higher temperatures. Recently, a continual ‘flow’ of actin in synapse-forming T cells from the periphery to near the centre of the synapse has been shown¹⁷. To determine whether this could be the cause of the shorter $t_{1/2}$ values, we employed the actin-depolymerizing drugs cytochalasin D and latrunculin A. These treatments greatly prolonged synaptic TCR–pMHC interactions, indicating that actin dynamics destabilizes TCR–ligand interactions either directly or indirectly (Fig. 2d). Under these conditions the $t_{1/2}$ is almost identical to that measured by SPR (4.23 s or 4.78 s *in situ*, versus 3.5 s *in vitro*) with the synaptic $t_{1/2}$ being slightly greater; this is expected because diffusion is more limited in that environment.

To estimate the affinity, we calculated the concentrations of the reactants and their products by first determining the local density (ρ) of total TCRs, pMHCs and TCR–pMHC complexes in a representative synapse. This was done on the basis of the average intensities of single-molecule fluorescence (Supplementary Fig. 21). To ensure a TCR labelling efficiency of at least 95%, we analysed synapses for 2.5 min or less at 24 °C (where the $t_{1/2}$ for scFv is about 50 min). With this image information (Fig. 3) we calculated the average local effective two-dimensional K_a (2D K_a) ($K_a = 1/K_d$) and, using the k_{off} determined above, the k_{on} ($k_{on} = K_a k_{off}$; Fig. 3a; see Methods). Hotspots of TCR binding are apparent, indicating that both local cellular parameters and the intrinsic chemistry of the interactions influence binding behaviour.

We also measured the affinities within individual TCR microclusters. To conduct our studies at higher temperatures we quantified the TCR occupancy of TCRs in individual TCR microclusters through their individual FRET yield (as measured by acceptor bleaching), which is independent of the degree of TCR labelling. This and a knowledge of the pMHC density in the bilayer allowed us to calculate the effective 2D K_d values (see Methods). The 2D K_d values for individual TCR microclusters varied significantly (about 250-fold), most probably because of locally active cellular parameters (Fig. 3b). We give the median *in situ* K_d values for comparison in Table 1.

To derive the effective synaptic three-dimensional K_d (3D K_d) of the interactions taking place within a TCR microcluster for comparison with the corresponding value measured by SPR, we converted the area for which binding was observed into the volume of the intermembrane space. To calculate the concentration we divided the density by the distance of 13.4 nm between the opposed membranes. This width is also the approximate maximum distance at which a TCR and pMHC could interact if fully extended and perpendicular to the plane of their respective membranes¹⁸. It is also consistent with electron microscopic

analyses of synapses¹⁹. For the interaction between 5c.c7 TCR and IE^K/MCC at 37 °C (at a density of 30 IE^K/MCC(C)-Cy5 μm^{-2}), this resulted in a median K_d of 4.8 μM , which is about 8.3-fold lower than the value determined *in vitro*, despite the about 12-fold accelerated decay rate that we observed *in situ*. Assuming steady-state conditions, we calculated a median synaptic k_{on} of $1.38 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$, an almost 100-fold increase compared with the SPR value at 37 °C (Fig. 3c and Table 1). We also determined the dependence of the TCR affinity on the density of ligands presented on the bilayer at 37 °C. Although constant at low and moderate pMHC densities, the median synaptic affinity decreased at ligand densities approaching that of the TCR, suggesting that some TCRs are refractory to binding (Fig. 3d and Table 1).

Synaptic binding properties also correlated with signalling potency: TCR binding was strongest for the agonist IE^K/MCC, followed by the weak agonist IE^K/T102S and the antagonists IE^K/T102G and IE^K/K99R (Fig. 3e). IE^K/T102S showed a single-exponential decay curve (Supplementary Fig. 1c) with a $t_{1/2}$ 2.4-fold lower (0.7 s) than that of IE^K/MCC. This difference is less than sevenfold, as seen with the equivalent SPR measurements (Table 1) and probably reflects the impaired signalling activity of this ligand (see below).

Of special interest with regard to helper T-cell recognition is the role of the CD4 co-receptor, which binds to a non-polymorphic region within MHC class II with a barely measurable affinity (250 μM or more)²⁰, yet has a main role in T-cell sensitivity⁷. A widely held view is that, to initiate signalling, simultaneous binding of TCR and CD4 to the same pMHC ligand directly recruits the TCR-proximal kinase p56^{lck} to the TCR through the association of p56^{lck} with CD4 (refs 21,22). Other models suggest that CD4 is associated with the TCR but does not bind the same MHC class II as its associated TCR^{7,22}. To distinguish between these two models, we first compared the average synaptic TCR–pMHC FRET yield in the absence and presence of CD4 blockade, because the FRET yield is related to the average synaptic TCR–pMHC affinity (Supplementary Fig. 12). CD4 blockade had little or no effect on the synaptic affinity between TCR and pMHC, both early and later in synapse formation. smFRET analysis revealed that CD4 blockade slightly stabilized interactions (Fig. 4a). An explanation could be that CD4 blockade attenuates T-cell signalling (Fig. 4b, c). This could dampen cellular motility, which destabilizes TCR–pMHC interactions. Thus we repeated CD4 blocking in the presence of pp2 (a p56^{lck} inhibitor) or cytochalasin D, which completely abrogated the CD4-mediated difference in the binding off-rate (Fig. 4d, e). Thus, the increased TCR–pMHC stability is due to signalling effects. Taken together, these results show that TCRs bind to ligands independently of CD4 and are not, as previously proposed, joined to CD4 molecules as a physical unit. TCR and CD4 could nevertheless still bind the same agonist pMHC simultaneously²¹, because independent and simultaneous binding events are not mutually exclusive, whether in solution or within the synapse. Because CD4 engagement boosts T-cell activation and sensitivity throughout synapse formation (Fig. 4b, c, and Supplementary Fig. 13), the function of CD4 seems likely to be that of signal reinforcement through its intracellular association with p56^{lck} once a TCR has engaged a viable ligand²¹.

Our data offer new insights into both how TCRs discriminate between different peptide-MHC ligands and also the unique challenges of finding and deriving the maximal signalling potential from even very rare agonists. In view of our data, the phosphorylation of TCR-associated CD3 signalling chains—that is, the first step in response to receptor engagement—is certain to result from frequent yet short-lived binding events. This means that, for the 5c.c7 TCR with its $t_{1/2}$ of about 100 ms, there could be roughly 3,000 separate receptor–ligand interactions in 1 min if ten ligands were engaged simultaneously in a synapse *in situ*, as is required for maximal calcium activation and stable synapse formation⁷. If the ligands were fully occupied and each interaction gave rise to a single signalling event, there would

be a significant rate of TCR signal transduction, equivalent to triggering about 10% of the TCRs in the cell.

How could rare pMHCs be kept occupied by TCRs, which are relatively sparse and have such unstable interactions? The explanation may lie in ‘protein islands’, distinct 10–200-nm membrane micro-domains highly enriched for particular membrane proteins^{12,13}. We find that clusters of TCRs are expressed on a subset of these islands, as also suggested by others¹⁴. Such TCR islands could efficiently ‘scan’ the surface of an adjacent cell and provide a high local density of TCRs surrounding a ligand to keep it continually engaged to generate the maximum possible signal. This is also consistent with cholesterol-depletion experiments in which TCR binding was no longer punctate: binding was decreased fivefold at higher and moderate pMHC densities and was no longer detectable at low densities (Supplementary Fig. 14). Receptor and ligand aggregation might also be facilitated by weak lateral interactions, substrate topography, membrane rigidities and line tension^{23,24}.

Differences in kinetic binding parameters could be substantially enhanced in a synaptic environment. Pre-oriented binding partners, molecular clustering and active T-cell scanning mechanisms would raise the frequency of otherwise rare molecular encounters. In contrast, interactions with a low $\Delta\Delta G$ might be even less stable as a result of cellular forces acting against them. In particular, we found that the 5c.c7-TCR of ‘average’ affinity was more severely destabilized (about 12-fold) than a higher-affinity 2B4-TCR (fourfold). This suggests that this unexpected activity in the synapse could help select for better-quality ligands and against weaker ones.

We have used a new imaging strategy to measure the binding of the TCR to its peptide–MHC ligand. Within an immunological synapse this shows that this very specialized but ubiquitous environment enhances the ability of otherwise very weak and rare ligands to drive important biological effects. With antibody pairs, for example, this FRET strategy could be used to characterize other molecular interactions as well. It seems likely that at least some of the binding characteristics found here will be shared with other cell-surface receptors on other cells, because molecules of this type generally have affinities and dissociation rates in the same range as TCRs^{10,11,18}.

METHODS SUMMARY

We take advantage of FRET to measure *in situ* the synaptic interactions between cell-bound TCRs and their cognate ligands, pMHC, embedded in a planar lipid bilayer. We engineered a site-specifically labelled TCR-reactive scFv to tag cell-surface-located TCRs. When the TCR is bound to its ligand, its associated scFv brings its label (FRET donor) close enough to the FRET acceptor dye attached to the MHC-embedded peptide to give rise to a FRET signal. We recorded smFRET to measure the $t_{1/2}$ values of synaptic TCR–pMHC interactions. We combined smFRET and bulk FRET measurements to determine synaptic K_d values and k_{on} values. We applied antibody-mediated blockade of CD4 to assess its contribution to TCR–pMHC binding.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Davis MM, et al. T cells as a self-referential, sensory organ. *Annu. Rev. Immunol.* 2007; 25:681–695. [PubMed: 17291190]
2. Grakoui A, et al. The immunological synapse: a molecular machine controlling cell activation. *Science.* 1999; 285:221–227. [PubMed: 10398592]
3. Monks CR, Freiberg BA, Kupfer H, Sciaky N, Kupfer A. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature.* 1998; 395:82–86. [PubMed: 9738502]
4. Corr M, et al. T cell receptor-MHC class I peptide interactions: affinity, kinetics, and specificity. *Science.* 1994; 265:946–949. [PubMed: 8052850]
5. Matsui K, Boniface JJ, Steffner P, Reay PA, Davis MM. Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc. Natl Acad. Sci. USA.* 1994; 91:12862–12866. [PubMed: 7809136]
6. Krogsgaard M, et al. Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. *Mol. Cell.* 2003; 12:1367–1378. [PubMed: 14690592]
7. Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. Direct observation of ligand recognition by T cells. *Nature.* 2002; 419:845–849. [PubMed: 12397360]
8. Sykulev Y, Joo M, Vturina I, Tsomides TJ, Eisen HN. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity.* 1996; 4:565–571. [PubMed: 8673703]
9. Bell GI. Models for the specific adhesion of cells to cells. *Science.* 1978; 200:618–627. [PubMed: 347575]
10. Dustin ML, Ferguson LM, Chan PY, Springer TA, Golan DE. Visualization of CD2 interaction with LFA-3 and determination of the two-dimensional dissociation constant for adhesion receptors in a contact area. *J. Cell Biol.* 1996; 132:465–474. [PubMed: 8636222]
11. Zhu DM, Dustin ML, Cairo CW, Golan DE. Analysis of two-dimensional dissociation constant of laterally mobile cell adhesion molecules. *Biophys. J.* 2007; 92:1022–1034. [PubMed: 17085486]
12. Lillemeier BF, et al. TCR and LAT occur in separate membrane domains and concatenate during activation. *Nature Immunol.* 2010; 11:90–96. [PubMed: 20010844]
13. Lillemeier BF, Pfeiffer JR, Surviladze Z, Wilson BS, Davis MM. Plasma membrane-associated proteins are clustered into islands attached to the cytoskeleton. *Proc. Natl Acad. Sci. USA.* 2006; 103:18992–18997. [PubMed: 17146050]
14. Schamel WW, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *J. Exp. Med.* 2005; 202:493–503. [PubMed: 16087711]
15. Garcia KC, et al. Structural basis of plasticity in T cell receptor recognition of self peptide-MHC antigen. *Science.* 1998; 279:1166–1172. [PubMed: 9469799]
16. Wang J, et al. Atomic structure of an $\alpha\beta$ T cell receptor (TCR) heterodimer in complex with an anti-TCR Fab fragment derived from a mitogenic antibody. *EMBO J.* 1998; 17:10–26. [PubMed: 9427737]
17. Kaizuka Y, Douglass AD, Varma R, Dustin ML, Vale RD. Mechanisms for segregating T cell receptor and adhesion molecules during immunological synapse formation in Jurkat T cells. *Proc. Natl Acad. Sci. USA.* 2007; 104:20296–20301. [PubMed: 18077330]

18. van der Merwe PA, McNamee PN, Davies EA, Barclay AN, Davis SJ. Topology of the CD2–CD48 cell-adhesion molecule complex: implications for antigen recognition by T cells. *Curr. Biol.* 1995; 5:74–84. [PubMed: 7697352]
19. Choudhuri K, Wiseman D, Brown MH, Gould K, van der Merwe PA. T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. *Nature.* 2005; 436:578–582. [PubMed: 16049493]
20. Xiong Y, Kern P, Chang H, Reinherz E. T cell receptor binding to a pMHCII ligand is kinetically distinct from and independent of CD4. *J. Biol. Chem.* 2001; 276:5659–5667. [PubMed: 11106664]
21. Li QJ, et al. CD4 enhances T cell sensitivity to antigen by coordinating Lck accumulation at the immunological synapse. *Nature Immunol.* 2004; 5:791–799. [PubMed: 15247914]
22. Krogsgaard M, et al. Agonist/endogenous peptide-MHC heterodimers drive T cell activation and sensitivity. *Nature.* 2005; 434:238–243. [PubMed: 15724150]
23. Fricke GM, Thomas JL. Receptor aggregation by intermembrane interactions: a Monte Carlo study. *Biophys. Chem.* 2006; 119:205–211. [PubMed: 16226368]
24. Weikl TR, Lipowsky R. Pattern formation during T-cell adhesion. *Biophys. J.* 2004; 87:3665–3678. [PubMed: 15377531]

Figure 1. FRET as a sensor for TCR-ligand interactions

a, Composite model based on the TCR-pMHC and TCR-H57 Fab structures. J1-J3 indicate dye attachment sites. **b**, Cy3, Cy5 and corrected FRET channels before and after acceptor bleaching at 24 °C. Scale bar, 5 μm. **c**, **d**, FRET yields determined through donor recovery after receptor bleaching. Error bars represent s.e.m. ($n \geq 40$; temperature 24 °C, $\rho(\text{IE}^k/\text{peptide-Cy5}) = 120 \mu\text{m}^{-2}$). **c**, Ligand specificity of the FRET yield. **d**, Relationship between Cy3 (FRET donor)-Cy5 (FRET acceptor) distance and FRET yield. Cy3-Cy5 distances were estimated from **a**, excluding the length of the linker and dye. The dotted line in **d** equals theoretical synaptic FRET yield ($R_0 = 4.6 \text{ nm}$) with a maximum (roughly 20%) predicted from experiments shown in Supplementary Fig. 23b.

Figure 2. Synaptic off-rates measured through smFRET

a, High-abundance FRET donor J1-Cy3, single molecule IE^k/MCC(C)-Cy5 (acceptor), smFRET channel and overlaid single-molecule channels are indicated. The white arrows indicate a single FRET event co-localizing with a single FRET acceptor, and the yellow arrows a candidate event co-localizing with several FRET acceptors. The intensity bars show counts per pixel. Scale bar, 5 μ m. **b**, Decay plot of interactions between synaptic TCR (J1-Cy3) and IE^k/MCC(C)-Cy5 at 37 °C. Open circles, 2B4 TCR ($t_{1/2}$ = 723 ms); filled circles, 5c.c7 TCR ($t_{1/2}$ = 109 ms). Error bars represent the standard deviation of the mean of the values as determined with different acquisition delays (for example 500, 1,000 and 2000 ms). **c**, Temperature spectrum of measured $t_{1/2}$ values of interactions between 5c.c7

and IE^k/MCC and between 2B4 TCR and IE^k/MCC (*in vitro:in situ* $t_{1/2}$ ratios are shown in grey). **d**, Interactions between 5c.c7 TCR and IE^k/MCC at 29.5 °C are prolonged on disruption of cortical actin through cytochalasin D and latrunculin A.

Figure 3. Quantitative image analysis yields effective synaptic 2D K_d , 2D K_a and 2D k_{on}
a, Synaptic density plots of TCR, IE^k/MCC and TCR-IE^k/MCC result in a 2D K_a plot (white print) and 2D k_{on} plot (red print). Dashed line, synapse boundary. Scale bar, 5 μm . **b**, 2D K_d distribution was measured for 5c.c7 TCR-IE^k/MCC microclusters at 37 °C. Values on the x axis show the upper interval boundary. The median value is 38.8. **c**, 3D k_{on} values were determined in 5c.c7 TCR-IE^k/MCC microclusters and by SPR. *In situ*:*in vitro* k_{on} ratios and *in vitro*:*in situ* K_d ratios are shown. **d**, 3D K_d values are shown for 5c.c7 TCR microclusters interacting at 37 °C with bilayers containing IE^k/MCC(C)-Cy5 at the indicated densities. **e**, 3D K_d , 3D k_{on} and $t_{1/2}$ values were measured at 24 °C in 5c.c7 TCR microclusters in contact with the indicated ligands.

Figure 4. Effect of CD4 on TCR signalling and ligand binding

a, Decay of interactions between synaptic 5c.c7 TCR and IE^k/MCC at 28 °C in the absence (black) and presence (red) of CD4 antibody blockade. **b**, Calcium signalling at 28 °C in the absence (black) and presence (red) of CD4 antibody blockade. **c**, T-cell proliferation (as measured by [³H]thymidine incorporation into naive T cells in response to varying doses of antigenic peptide) in the absence (black) and presence (red) of CD4 antibody blockade. Open symbols, with H57 scFv; filled symbols, without H57 scFv. Error bars represent the standard deviation of the mean for data sets measured in triplicate. **d**, CD4 blockade did not affect the synaptic $t_{1/2}$ of the 5c.c7 TCR-IE^k/MCC complex at 28 °C after inhibition of the

activity of p56^{lck} with pp2. **e**, CD4 blockade did not affect the synaptic $t_{1/2}$ of the 5c.c7 TCR–IE^k/MCC complex at 29.5 °C after disruption of the actin cortex with cytochalasin D.

Summary of measured binding constants

Table 1

Ligand	Temperature (°C)	Density* (molecules μm ⁻²)	Median [†] <i>in situ</i> 3DK _d (μM)	<i>In vitro</i> [‡] K _d (μM)	<i>In situ</i> t _{1/2} (s) [k _{off} (s ⁻¹)]	<i>In vitro</i> [‡] t _{1/2} (s) [k _{off} (s ⁻¹)]	Median [‡] <i>in situ</i> 3DK _{on} (s ⁻¹ M ⁻¹)	<i>In vitro</i> [‡] k _{on} (s ⁻¹ M ⁻¹)
IE ^k /MCC	24.0	30	4.26	22.9	1.68 [0.41]	5.77 [0.120]	9.75 × 10 ⁴	3.31 × 10 ³
IE ^k /MCC	28.0	30	4.33	24.7	1.25 [0.55]	4.0 [0.173]	1.26 × 10 ⁵	5.49 × 10 ³
IE ^k /MCC, CD4 block	28.0	30			1.63 [0.43]			
IE ^k /MCC, pp2	28.0	30			1.57 [0.44]			
IE ^k /MCC	29.5	30			0.77 [0.90]			
IE ^k /MCC, Cyt D	29.5	30			4.23 [0.163]			
IE ^k /MCC, Lat A	29.5	30			4.78 [0.144]			
IE ^k /MCC	33.0	30	4.02	27.7	0.405 [1.7]	2.28 [0.305]	4.22 × 10 ⁵	1.12 × 10 ⁴
IE ^k /MCC	37.0	30	4.8	39.8	0.109 [6.36]	1.24 [0.559]	1.38 × 10 ⁶	1.40 × 10 ⁴
IE ^k /MCC	37.0	6	4.16		0.138 [5]		1.20 × 10 ⁶	
IE ^k /MCC	37.0	150	8.67		0.111 [6.25]		7.20 × 10 ⁵	
IE ^k /T102S	24.0	30			0.7 [1.02]	0.82 [0.838]		
IE ^k /T102S	24.0	139	18.2	206			5.59 × 10 ⁴	4.05 × 10 ³
IE ^k /T102S, Lat A	24.0	30			0.99 [0.7]			
IE ^k /T102G	24.0	175	42.6					
IE ^k /K99R	24.0	175	208					

Cyt D, cytochalasin D; Lat A, latrunculin A.

* Where applicable.

[†] Values measured in individual TCR microclusters.

[‡] Values obtained by SPR.