Ligand-engaged TCR is triggered by Lck not associated with CD8 coreceptor

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The earliest molecular events in T-cell recognition have not yet been fully described, and the initial T-cell receptor (TCR)-triggering mechanism remains a subject of controversy. Here, using total internal reflection/Forster resonance energy transfer microscopy, we observe a two-stage interaction between TCR, CD8 and major histocompatibility complex (MHC)-peptide. There is an early (within seconds) interaction between CD3ζ and the coreceptor CD8 that is independent of the binding of CD8 to MHC, but that requires CD8 association with Lck. Later (several minutes) CD3ζ–CD8 interactions require CD8–MHC binding. Lck can be found free or bound to the coreceptor. This work indicates that the initial TCR-triggering event is induced by free Lck.
Antigen recognition by the T-cell receptor (TCR) is the first step in T-cell activation, and the paramount event for their development and functions. At the start of the signalling cascade, the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 signalling subunits of the TCR are phosphorylated by the Src-family kinase (SFK) Lck and, to a lesser extent, Fyn, and then bound by another kinase called ZAP70 (refs 1–3). After ZAP70 binds to CD3, the coreceptors CD4 or CD8, which are associated with the SFK Lck, become associated with the TCR–CD3 complex and bind to the major histocompatibility complex (MHC). This stabilizes the TCR–MHC-peptide (MHCp) interaction (at least in the case of CD8 (ref. 4)), and Lck continues the phosphorylation of CD3 elements, ZAP70 and the many other downstream targets. Freshly isolated T cells, but not T cell lines, show partial phosphorylation of CD3ε with bound but non-phosphorylated ZAP70 (ref. 5). This condition is thought to represent stimulation by self-MHCp in vivo.

Although TCR triggering has been extensively studied, the precise timing of signals downstream of the TCR is as yet poorly understood. TCR microclusters, in which signalling occurs, form at the immunological synapse (IS) within seconds of MHCp recognition. Using MHC complexes containing an antigenic peptide that is biologically inert until exposed to ultraviolet light, Huse et al. developed a high-resolution temporal analysis of signalling. Phosphorylation of the adaptor molecule linker for activation of T cells (LAT) was observed within 4 s, and diacylglycerol production and calcium flux was observed after 6–7 s (ref. 8). A recent biophysical study on TCR and CD8 binding to MHCp demonstrated that the initial binding of TCR to MHCp induces, in a SFK-dependent manner, the binding of CD8 to the MHCp, leading to the question of how the TCR–CD3 complex is initially phosphorylated by an SFK before CD8 and the associated Lck have been recruited to the TCR–CD3 complex.

CD8 coreceptor is expressed on cytotoxic T lymphocytes and their double-positive thymic progenitors, where its main function is to augment the sensitivity and response of T cells to cognate MHCp ligands. It is generally accepted that the ability of the coreceptor to enhance T-cell responses is due to two main effects: (i) Binding of CD8 to MHC class I (MHC) molecules helps stabilize weak TCR–MHC interactions; (ii) the recruitment of Lck, which is bound to the cytoplasmic tail of the coreceptor, to the TCR complex upon coreceptor binding to the MHC, thereby enhancing the initiation of TCR signalling. Lck consists of N-terminal sequences that mediate its myristoylation, palmitoylation, or associated with the CD8 or CD4 coreceptors anchored to the plasma membrane through myristoylation and N-terminal sequences that mediate its myristoylation, enhancing the initiation of TCR signalling. Lck consists of the TCR complex upon coreceptor binding to the MHC, thereby enhancing the initiation of TCR signalling.

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### Results

#### Antigen induced CD8β–CD3ε interaction

We first assessed the relationship between CD8 recruitment to the immune synapse and its ability to bind to MHCp by analysing the synapses formed between OT-I T-cell hybridomas expressing CD3ε–CFP and CD8αβ–YFP (hybridoma cell line hence referred to as OT-I.ZC.8(Y)) cells, and a surrogate antigen-presenting cell line: CHO cells expressing either inducible wild-type sCh2-Kb–, OVA or a D227K/E229K mutant that cannot bind to CD8 (sCh2-Kb–OVA–CD8mut). These sc-MHCp constructs were described previously. The OT-I TCR recognizes the agonist OVA peptide (SIINFEKL) with high affinity, but not the VSV peptide (RGYVYQGL), when either is presented by H-2Kb.

We compared the intensity of CD8β–YFP fluorescence in the immune synapse, showing that the CD8 recruitment itself was not solely a result of TCR recognition of MHCp, but instead was driven by the CD8–MHCp interaction (Fig. 1a), as indicated by our earlier work. Because of the differences in timing between our previous measurements of FRET between TCR and coreceptors, and other’s analyses of early signalling events during T-cell antigen recognition, we decided to further characterize the TCR–CD8 interaction. The interaction between two molecules can be studied using FRET, which occurs only between molecules separated by 10 nm or less. After donor excitation, FRET leads, due to the energy transfer, to decreased donor fluorescence and increased acceptor fluorescence. We allowed CHO cells expressing either sc-H-2Kb–OVA or sc-H-2Kb–VSV to settle in a 37°C-heated chamber before the addition of OT-I.ZC.8(Y) cell line: CHO cells expressing either inducible wild-type sCh2-Kb–, OVA or a D227K/E229K mutant that cannot bind to CD8 (sCh2-Kb–OVA–CD8mut). These sc-MHCp constructs were described previously. The OT-I TCR recognizes the agonist OVA peptide (SIINFEKL) with high affinity, but not the VSV peptide (RGYVYQGL), when either is presented by H-2Kb.
cells. We monitored the interaction between CD3ζ-CFP and CD8β-YFP by live FRET efficiency (E-FRET) imaging during the process of antigen recognition. After stimulation with sc-H-2Kb-OVA-expressing cells, both CD3ζ-CFP and CD8β-YFP were recruited to the synapse, where the FRET signal between CD3ζ-CFP and CD8β-YFP began to increase strongly about 5 min after the beginning of the antigen-presenting cell (APC)–T-cell interaction (Fig. 1b) until it peaked at 10 min, remaining oscillating for another 10 min and then decreasing. No detectable FRET was found at any time when the T cells interacted with the non-stimulatory sc-H-2Kb-VSV-expressing CHO cells. These results agreed with previous studies from this lab, but we considered it likely that we were underestimating the timing and the potency of the interaction owing to technical limitations of the experimental setup, and to the fact that we were analysing the average FRET efficiency over the whole synapse. During the interaction of sc-H-2Kb-OVA-expressing CHO cells with 17β cells (T-cell hybridoma expressing OT-I TCR and CD8β) loaded with the Ca\(^{2+}\) fluorescent indicator Fluo-4, cytosolic Ca\(^{2+}\) concentration [Ca\(^{2+}\)] increased at the very moment that the initiation of the interaction was observed (Fig. 1b, right plot). This suggested that we were indeed missing early signalling events.

Looking closely at the FRET data in Fig. 1b, we noted that there appeared to be a slight rise in the E-FRET signal at the time of contact between the T cell and the APC, which continued until the main E-FRET increase at ~5 min. While its significance was questionable, we hypothesized that this could represent small localized regions of TCR–CD8 interaction that were ‘diluted’ in the analysis of the whole synapse. To improve the resolution of our imaging, we used artificial supported lipid bilayers as antigen-presenting surfaces, imaging the IS between the T cell and the bilayer presenting H-2Kb-peptide complex as well as ICAM-1 (Fig. 2a). We used a TIRF microscopy system that allowed us to increase spatial resolution by exciting only the fluorophores immediately adjacent to the glass–oil interface (z = 100 nm), in other words, imaging the immune synapse. The bilayers were fully functional as surrogate antigen-presenting surfaces, supporting full lipid motility, determined by fluorescence recovery after photobleaching (Supplementary Fig. 1a) and antigen-specific T-cell activation (determined by MHCP relocation in Supplementary Fig. 1b, Ca\(^{2+}\) flux in Supplementary Fig. 1c and total phosphotyrosine imaging in Supplementary Fig. 1d). We switched fluorophores to green and red fluorescent proteins (enhanced green fluorescent protein (eGFP) as donor and mCherry as acceptor) to be compatible with the TIRFM system. Thus, using a new hybridoma cell line expressing CD3ζ-eGFP and CD8β-mCherry, hence referred to as OT-I.ZG.8R, T cells stimulated by H-2Kb-OVA bilayers we were able, not only to reproduce the kinetics of our previous results in live and fixed cells, but also to detect a weak but significant increase in FRET efficiency in the earliest time point analysed (1 min) (Fig. 2b,c). This FRET signal was not observed in T cells interacting with bilayers presenting the non-stimulatory H-2Kb-VSV complexes. This early FRET was observed in small localized clusters. This likely corresponds to the very early moments of antigen recognition, taking into account that it takes about 30–60 s for the settling cells to reach the bilayer. After this initial CD3ζ–CD8β interaction, a stronger interaction occurred after about 5 min. These results clearly indicate a two-stage interaction process.

**CD8β–CD3ζ interaction is a two-stage process.** A recent study also suggests biphasic TCR and CD8 interactions with peptides...
Lck–CD8 interaction is required for initial CD8 recruitment. To test whether CD8 needed to be bound to Lck to interact with the TCR–CD3 complex after antigen recognition, we imaged early and late FRET signals during antigen recognition by OT-1 T-hybridoma cells expressing CD3\(\alpha\)-eGFP, CD8\(\beta\)-mCherry and CD8 with a mutation of the intracellular CxCP motif that mediates the interaction with Lck (hence referred to as OT-1.ZG.8\(\beta\)-MHC\(\mu\)mutR)\(^{23,28,32,33}\). The C227KC229P motif was mutated to S227KS229P (ref. 33). This experiment showed that the Lck–binding mutation completely abrogated the early interaction between CD3\(\alpha\)-eGFP and CD8\(\beta\)-mCherry as well as the late phospho-Tyr signalling (Fig. 3a,b). This suggests that the Lck interaction with CD8 was required to bring CD8 close to the TCR–CD3 complex at an early time point. The interaction between MHC1 and CD8 is strong enough to overcome this effect at later time points, as indicated by the recovery of the second interaction stage (Fig. 3a).

CD8–Lck and MHCp interactions required for two-stage kinetics. Our FRET data strongly suggest that CD8 interactions with TCR–CD3 occur in two stages, with the early (<1 min) stage requiring CD8 binding to Lck, but not to MHC, and the late (>5 min) stage requiring CD8 binding to MHC, but not to Lck. We validated the relevance of this finding using the adhesion frequency assay for analysis of two-dimensional binding described previously\(^{9,34,35}\). In brief, an OT-1 T-hybridoma cell with various combinations of transduced signalling proteins and a red blood cell (RBC) bearing H-2K\(b\)-OVA were aspirated by respective pipettes and driven in and out of contact with controlled area and duration. Adhesion was defined as stretching of the RBC on T-cell retraction\(^{5}\). This contact–retraction cycle was repeated 50 times for a given contact duration on each cell pair. We performed two-dimensional binding studies with the three different hybridomas expressing interaction between the three molecules. Thus the kinase activity of the first stage occurs before CD8 has had a chance to associate with the MHC1. Since the SFK Lck is thought to be associated with the coreceptor, the mechanism for the initial phosphorylation step is unclear\(^{9}\). Using a T-cell hybridoma expressing OT-I TCR with CD3\(\alpha\)-eGFP, CD8\(\alpha\) and a S101A/K103D CD8\(\beta\) mutant that cannot bind to MHC1, CD8\(\beta\)-MHC\(\mu\)mutR\(^{23,28,32}\). We observed an early CD3–CD8 FRET signal in T cells expressing the CD8 mutant that cannot bind to MHC1, when exposed to MHCp complexes, and this FRET signal was comparable to the signal observed for T cells expressing wild-type CD8 (Fig. 3a). In contrast, CD8 unable to bind MHC1 did not interact with CD3 at 15 min, and did not enable T-cell activation (Fig. 3a,b). To further confirm the importance of CD8 binding to MHC1 for the late FRET signals, we tested the activation state of the cells (15 min) by total phospho-Tyr staining and compared total phospho-Tyr levels in cells expressing wild-type CD8 in response to wild-type MHC1 monomers or with chimeric monomers consisting of mouse 5.12 heavy chain and human \(\zeta\) domain that cannot bind to mouse CD8 (H-2K\(b\)-OVA-HLA-A\(^*\)02:01)\(^{9}\) presented on the bilayers with cells expressing CD8 mutant that cannot bind MHC1 (Fig. 3c). In addition, we used primary OT-I cytotoxic T lymphocyte (CTL) cells plus or minus CD8-blocking antibodies in bilayers presenting wild-type MHC1 molecules or MHC1 molecules that cannot bind CD8 (Fig. 3d). When the blocking anti-CD8 antibody (Ab) was present, or in response to MHC1 mutant, total phospho-Tyr activity was as low as when the non-stimulatory VSV peptide was presented (Fig. 3c,d).

**Figure 2** | Induction of TCR–CD8 FRET by an agonist on artificial lipid bilayers. (a) Scheme of supported lipid bilayers used as artificial antigen presenting surfaces. (b) OT-I hybridomas expressing CD8\(\alpha\) wild-type, CD3\(\alpha\)-eGFP and CD8\(\beta\)-mCherry (OT-I.ZG.8\(\beta\)) were added to lipid bilayers containing either H2-K\(b\)-OVA or H2-K\(b\)-VSV monomers and ICAM-1, fixed at the indicated time points and imaged by TIRFM at the immune synapses. Images shown in green represent CD3\(\alpha\)-eGFP, CD8\(\beta\)-mCherry in red and FRET efficiency in false colour scale. Images are representative of three different experiments. Scale bar, 5 \(\mu\)m. (c) FRET efficiency analysis of immune synapses from OT-I.ZG.8\(\beta\)R interacting with lipid bilayers containing H2-K\(b\)-OVA (circles). Mean \pm\ s.e.m. of 1 min \(n = 18\), 2 min \(n = 33\), 3 min \(n = 18\), 5 min \(n = 45\), 10 min \(n = 50\) and 15 min \(n = 52\). Or H2-K\(b\)-VSV bilayers (squares), mean \pm\ s.e.m. of 1 min \(n = 12\), 2 min \(n = 15\), 3 min \(n = 13\), 5 min \(n = 21\), 10 min \(n = 14\) and 15 min \(n = 13\). Unpaired \(t\)-test. \(*P < 0.01\) and **\(P < 0.001\).

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Presented by MHC1\(^{9}\). In the first step, TCR binds MHCp, causing SFK activity that is a pre-requisite for the second stage, when CD8 binds MHC1\(^{9}\). The CD8–MHC1 interaction stabilizes the
either wild-type CD8 molecules, or CD8 with abrogated binding to Lck or MHC. The kinetics of their interactions with K\(^\beta\)-OVA, were expressed as the normalized adhesion bonds \(< n > / m_{3+\text{MHC}}\). Plots of \(< n > / m_{3+\text{MHC}}\) of cells expressing OT-I TCR with CD3\(^\zeta\), CD8\(x\) and CD8\(\beta\) showed two-stage kinetics, whereas plots from cells expressing either CD8\(x\)-CxCP\(_{\text{mut}}\) (Fig. 4a) or CD8\(\beta\)-MHC\(_{\text{mut}}\) (Fig. 4b) showed single-stage kinetics. This confirmed that the Lck interaction with CD8 was required to bring CD8 close to the TCR, and that the CD8–MHCI interaction was responsible for the second binding stage that results in the late FRET peak.

To investigate TCR triggering in primary T cells, CTLs were prepared from OT-I TCR transgenic mouse splenocytes after activation by OVA peptide with addition of interleukin (IL)-2. CTLs were transduced with a biosensor for CD3 ITAM phosphorylation consisting of mCherry fused to the tandem SH2 domains of ZAP70 (mCherry-tSH2(ZAP70))\(^{36}\). In resting T cells ZAP70 is located in the cytosol, but upon TCR activation it relocates to the plasma membrane through association of its SH2 domains to the phosphorylated ITAMs of the CD3 complex. This translocation can be detected by fluorescence microscopy upon T-cell activation following crosslinking with anti-CD3 + anti-CD8 (Fig. 5a). Due to the optical characteristics of TIRF illumination, this sensor is extremely useful to analyse T-cell activation at the immune synapse. After adding OT-I CTL expressing the mCherry-tSH2(ZAP70) biosensor to lipid bilayers presenting specific MHCP (H-2K\(^\beta\)-OVA), we observed consistent presence of mCherry-tSH2(ZAP70), indicating CD3 ITAM phosphorylation, in the immune synapse. With bilayers presenting the chimeric variant that cannot bind to CD8 (H-2K\(^\beta\)-OVA-HLA-A\(^*\)02:01), early but transient accumulations of ZAP70 were noted (Fig. 5b). When primary OT-I CTLs were analysed by western blot for specific phosphorylation of CD3\(^\zeta\) and for Ca\(^{2+}\) flux, after stimulation with H-2K\(^\beta\)-OVA tetramer, we confirmed that the initial CD3\(^\zeta\) phosphorylation and Ca\(^{2+}\) transients were high and sustained (Fig. 6). When the tetramers were unable to bind to CD8 there was only a small initial peak of CD3\(^\zeta\) phosphorylation, and a Ca\(^{2+}\) flux that was not sustained over time. CD3\(^\zeta\) phosphorylation was completely inhibited by addition of the SFK inhibitor PP2 (Fig. 6; Supplementary Fig. 3a). Together these data confirm, as expected, the importance of CD8–MHC interaction for the stabilization of the trimolecular complex and for maintenance of the TCR signalling process. More interestingly, these data also demonstrate an initial SFK-dependent phosphorylation of ITAMs on the CD3 complex that is independent of the coreceptor binding to MHC.

**A small amount of Lck is enough to phosphorylate the TCR.** Several lines of evidence indicate that the initial phosphorylation of CD3\(^\zeta\) ITAMs and ZAP70 is mediated by Lck and/or Fyn\(^{37–39}\). Lck-deficient mice have stronger defects in most aspects of signalling than Fyn-deficient mice\(^{40}\), yet the mechanism of the
process that initiates the TCR signalling cascade is still obscure\(^3\). Therefore, we knocked down Lck expression in OT-I primary CTLs using specific small hairpin RNA. After a decrease in Lck protein expression of about 60%, we were still able to observe CD3\(\zeta\) phosphorylation to the same degree as control-treated cells after H\(\text{2-Kb}\)-OVA tetramer stimulation (Supplementary Figs 2 and 3a,e). These data suggest either that Fyn is compensating for Lck absence, or that only a small pool of the total Lck is responsible for the initial phosphorylation.

**Free Lck is responsible for initial TCR–CD3 phosphorylation.** Our data strongly indicate that a free, rather than coreceptor-bound, pool of Lck initiates CD3 phosphorylation. To further

**Figure 4 | Adhesion frequency assay.** (a) Frequency of adhesion was normalized to the amount of pMHC presented \(\langle n\rangle/m_{\text{pMHC}}\) and plotted against time \(t\) for OT-I hybridomas expressing CD8\(\alpha\) wild-type and CD8\(\beta\)-wild-type (closed circles), or CD8\(\alpha\)-CxCP\(\mu\) and CD8\(\beta\) wild-type (closed squares) T cells interacting with RBCs bearing 4 and 7 OVA-H-2K\(\beta\) per mm\(^2\), respectively, at 37°C. Controls with CD8 wild-type cells pretreated with a blocking antibody against TCR (open diamond), or using RBCs bearing 93 VSV-H-2K\(\beta\) per mm\(^2\) (open triangle) were conducted. Representative data from three repeated experiments are shown. (b) \(\langle n\rangle/m_{\text{pMHC}}\) versus \(t\) data of OT-I hybridomas expressing CD8\(\alpha\) wild-type and CD8\(\beta\)-MHC\(\mu\) (closed triangles) T cells interacting with RBCs bearing 23 OVA-H-2K\(\beta\) per mm\(^2\). Controls with CD8 wild-type cells pretreated with a blocking antibody against TCR (open diamond), or using RBCs bearing 93 VSV-H-2K\(\beta\) per mm\(^2\) (open triangle) were conducted. Data are representative of three experiments.

**Figure 5 | CD8/MHCp interaction is required for sustained signaling through the TCR.** (a) Nuclei of OT-I primary CTL expressing mCherry-tSH2(ZAP70) were stained with the live cell-imaging-compatible Hoechst 33342 and then incubated with a mixture of biotinylated anti-CD3\(\epsilon\) and anti-CD8\(\beta\) antibodies and imaged under a deconvolution microscope. Top row shows the localization of mCherry-tSH2(ZAP70) before (left) and after the addition of crosslinking streptavidin (right). Higher detail in zoomed boxes in false colour. Bottom, graphs showing decrease in cytosolic fluorescence after streptavidin addition (left) in selected regions, or ratio between membrane and cytosolic fluorescence (right). Data are representative of at least three different experiments. Scale bar, 5 \(\mu\)m. (b) OT-I primary CTL cells expressing mCherry-tSH2(ZAP70) were added to lipid bilayers containing either H\(\text{2-K}\^\(\beta\)-OVA or H\(\text{2-K}\^\(\beta\)-OVA-HLA-A*02:01 monomers and ICAM-1, and analysed by live cell TIRFM at the immune synapses. Representative time points are shown in the top rows where the cells can be seen by transmitted light and the recruitment of mCherry-tSH2(ZAP70) is shown as a false colour overlayed image. Bottom graph, normalized mean mCherry-tSH2(ZAP70) fluorescence at the immune synapse in bilayers containing either H\(\text{2-K}\^\(\beta\)-OVA (red) or H\(\text{2-K}\^\(\beta\)-OVA-HLA-A*02:01 (blue). Data are representative of three experiments.
transgene expression with tetracycline successfully overcomes the
right-hand plot, cells were loaded with Ca\(^{2+}\) and

immune synapse, we expressed wild-type or C20A/C23A mutant

contribution of free Lck to TCR antigen recognition at the

substantial CD3\(^{\zeta}\) interactions with lipid bilayers presenting H2-K\(^{b}\)-OVA and

CTLs and monitored, using TIRFM, its localization during

Lck fused to mCherry or eGFP, respectively, in LckOFF OT-I

(0.51 \pm 0.01 versus 0.50 \pm 0.01, respectively) or at any time point

analysed when the bilayers contained VSV (Fig. 8b). These results

strongly suggest that the free Lck initially interacts with TCR
during antigen recognition, whereas coreceptor-bound Lck is

recruited to the TCR/CD3 complex at later time points.

**Discussion**

Early T-cell signalling events such as LAT phosphorylation or
calcium flux take place within a few seconds\(^8\). Performing higher-
spatial/temporal resolution experiments using a supported lipid
bilayer system as a surrogate antigen-presenting surface, we have
been able to image TCR–CD8 FRET in small regions within the
IS. These were too localized to show a signal above the
background when FRET signals were averaged over the whole
synapse, explaining why they were not noticed before in regular
live or fixed epifluorescence experiments\(^24\). A recent study on
TCR and CD8 binding to MHCp showed that there is a two-stage
interaction process in which first TCR interacts with MHCp and,
after a SFK-mediated activity, there is a stabilization phase
charged by CD8–MHCp binding\(^29\). This was identified using
blocking antibodies against CD8 and the SFK inhibitor PP2, where
only the first phase of the interaction takes place\(^9\).

Our new FRET/TIRF data show a small but significant increase in
FRET signal within 1 min of adding cells to the imaging
chamber, followed by a second, stronger, increase after about
5 min. This suggests that the early FRET signal represents CD8
brought passively to the TCR by binding of Lck to phospho-CD3
or phospho-ZAP70. The later, stronger FRET was likely due to
stabilization of the TCR–MHCp complex by CD8 binding to

investigate the role of free Lck in initiation of TCR signalling, we
used Rag\(^1\)\(^{\Delta/\Delta}\) OT-I mice in which Lck expression was controlled by the induction of a tetracycline-responsive transgene\(^41\).

These mice are on an Lck\(^{-/-}\) background, and induction of
transgene expression with tetracycline successfully overcomes the
lack of endogenous Lck, restoring normal thymopoesis\(^41\).
Withdrawal of the mice from tetracycline for at least 7 days
(LckOFF) is sufficient to remove all residual protein\(^42\). We
generated CTL from the LckOFF mice, and stimulated them with
the agonist H-2K\(^{b}\)-OVA tetramers. In this system, where
there is no Lck present in the cells, there was no detectable

phosphorylation after antigen recognition.

Figure 6 | Src kinase family activity is responsible of TCR
phosphorylation after antigen recognition. Primary OT-I CTL were
pretreated or not with the general Src kinase inhibitor PP2 (20 mM) and
then stimulated with either H2-K\(^{b}\)-OVA or H2-K\(^{b}\)-OVA-HLA-A\(^{\zeta}\)-02:01
tetramers for the indicated times. CD3\(^{\zeta}\) phosphorylation was analysed by
immunoblot. Quantification of band intensities is shown in the left plot.
In the right-hand plot, cells were loaded with Ca\(^{2+}\)-sensitive dye Fluo-4 and
imaged after the addition on top of bilayers containing either H2-K\(^{b}\)-OVA or
H2-K\(^{b}\)-OVA-HLA-A\(^{\zeta}\)-02:01 monomers plus ICAM-1, as described in the
Methods section. Data are representative of four experiments. Data are
representative of three experiments.

Figure 7 | Free Lck is responsible for initial TCR phosphorylation.
(a) OT-I Rag\(^{1/\Delta}\) wild-type or Lck\(^{-/-}\) CTL were stimulated with
H2-K\(^{b}\)-OVA tetramers for the indicated times. CD3\(^{\zeta}\) phosphorylation and
Lck expression were analysed by immunoblot. Data are representative of
three experiments. (b) OT-I Rag\(^{1/\Delta}\) Lck\(^{-/-}\) CTL cells were transduced
with Lck-eGFP wild-type or with the mutant Lck(C20.23A)-eGFP and then
stimulated with H2-K\(^{b}\)-OVA tetramers for the indicated times. CD3\(^{\zeta}\)
phosphorylation and total Lck expression was analysed by immunoblot.
Data are representative of two experiments.
MHCI, because of the absence of induction of the FRET signal or Tyr-phosphorylation in cells expressing a mutant CD8 that cannot bind MHCI. This suggested that the interaction operative at early stages goes away over time. Moreover, when cells expressed a CD8 mutant that cannot bind Lck, there was only a low FRET signal, demonstrating that the Lck interaction with CD8 was required to bring CD8 close to the TCR. It might be possible that the interaction between MHCI and CD8 is strong enough to overcome this effect at later time points, as suggested by the recovery of the second interaction stage. These results were supported using the same biophysics approach conducted by Jiang et al. in their work. Using OT-I T-cell hybridomas we were able to detect a two-stage interaction kinetic between TCR–CD8 and MHCP only when cells expressed wild-type CD8 molecules. When the cells expressed either the Lck–CD8-binding mutant or MHC–CD8-binding mutant, only the first stage of the interaction was detectable.

It is commonly accepted that CD8 is recruited to the signalling TCR by its MHCP interaction. However, if the CD8–MHCP interaction does not happen until after TCR ITAMs are phosphorylated, how is the initial CD8 recruitment produced? One possibility supported by our data is that passive diffusion would allow Lck to bind to the phosphorylated ITAMs on the TCR, or even directly to phospho-ZAP70, thus bringing any coreceptor-associated Lck passively to the TCR. An earlier study with chimeras of CD4–Lck showed that mutation of the Lck SH2

Figure 8 | Lck recruitment analysis. (a) OT-I Rag1−/− CTL were transduced with wild-type mCherry-fused Lck (top row) or mutant Lck-C20.23A-eGFP (middle row), added to lipid bilayers containing H2-Kb-OVA monomers and ICAM-1 (top two rows), or ICAM-1 only (bottom row), and the immune synapses were imaged under TIRF microscopy. Cells can be followed by transmitted light. Lck intensity is shown in false colour at selected time points. Scale bar, 5 μm. Bottom, intensity line profile of cells interacting with bilayers containing H2-Kb-OVA monomers and ICAM-1 (green) or ICAM-1 only (blue). Data are representative of three experiments. (b) Double colocalization analysis of immune synapses from OT-I.ZG.8.Lck.C.8β.Lck C2023A.R cells interacting with lipid bilayers containing H2-Kb-OVA (left) or H2-Kb-VSV (right) plus ICAM-1. Scale bar, 5 μm. Means ± s.e.m. of two separate experiments of Pearson’s coefficient between CD3ζ-eGFP and Lck-C20.23A-mCherry (red squares) or CD3ζ-eGFP and CD8α-Lck-Cerulean (blue circles) were plotted.
domain in the chimera reduced the ability to restore thymopoiesis independently of the kinase activity, suggesting a protein–protein interaction role for Lck44.

Although Lck phosphorylation of TCR ITAMs is one of the earliest detected events in T-cell activation, it still remains unsolved. Briefly, Lck activity is controlled by a balance between phosphorylation of two main tyrosine residues, Tyr397, known to promote an open active conformation, and Tyr505, known to promote a closed inactive conformation32,44. Conflicting data on this subject are available. Most traditional biochemical studies showed no changes on the phosphorylation level of Lck after TCR stimulation, so Lck could be accessible to very subtle regulatory mechanisms without the need for acute changes in Tyr397 and Tyr505 phosphorylation and conformational alterations, as FRET studies with an Lck sensor claimed45. On the other hand, more fine measurements with the same sensor, analysing its studies with an Lck sensor claimed45. On the other hand, associated with the TCR–CD3 complex, and phosphorylates upon TCR binding to MHCp, non-CD8-bound Lck becomes localized to the plasma membrane, with some Lck also associated with CD8. This was able to restore induction of TCR phosphorylation and stimulation. Given these findings, we propose a model in which recognition than did coreceptor-associated Lck. This was similar to previous reports44 that up to 40% of total Lck present in unstimulated naïve T cells and thymocytes was constitutively active, consistent with the data obtained with the fluorescent Lck reporter construct45,46. This substantial, pre-existing pool of active Lck molecules in the membrane of resting T cells might be sufficient to mediate phosphorylation of the TCR. To address this question, we have found that a mutant Lck (C397A/C505A) that cannot bind to CD8 was able to restore induction of TCR phosphorylation and showed a higher colocalization index with TCR after MHCp recognition than did coreceptor-associated Lck. This was similar to the effect of wild-type Lck in Lck-deficient cells after T-cell stimulation. Given these findings, we propose a model in which before TCR interaction with MHCp, some Lck is associated with the plasma membrane, with some Lck also associated with CD8. Upon TCR binding to MHCp, non-CD8-bound Lck becomes associated with the TCR–CD3 complex, and phosphorylates CD3z ITAMs and then ZAP70, which binds to the phosphorylated CD3z ITAMs. The SH2 domain of CD8-bound Lck then interacts with phospho-ZAP70, pulling CD8 into proximity with the MHCp-interacting TCR47,48. The extracellular immunoglobulin domains then bind to the MHCp, stabilizing the TCR–MHCp interaction.

Methods

Mice. B6 mice were bred at NUS. All experimental procedures performed in Singapore were approved by the institutional animal care and use committees of the National University of Singapore. OT-I, OT-I Rag1−/− mice all on the B6 background were bred at The Scripps Research Institute and were maintained in accordance with the Animal Care and Use Committee of The Scripps Research Institute. OT-I Rag1−/−; Lck−/− spleens were provided by Rose Zamoyska (School of Biological Sciences, University of Edinburgh). Mice of either sex were used between 5 and 8 weeks of age. Mice were age and sex matched in each experiment.

Constructs and cells. CHO cells expressing the tetracycline repressor (Invitrogen) under blasticidin selection (10 μg ml−1) were grown in Ham’s F12 media with 10% (v/v) FCS, 100 U ml−1 of penicillin, 10 mg ml−1 of streptomycin, 0.3 mg ml−1 of hygromycin, 418 0.8 mg ml−1. CTLs were prepared from OT-I transgenic mice spleen cell suspensions. RBCs were lysed with ACK buffer, and the cell suspension was cultured in an upright T75 flask, in complete RPMI (10% fetal bovine serum, 50 mM β-ME, 2 mM L-glutamine, 100 U ml−1 penicillin and 10 mg ml−1 streptomycin) plus 10 U ml−1 IL-2 plus 10 μM OVA peptide for 48–72 h. The cells were washed to remove peptide and resuspended in fresh medium with IL-2. CTLs were used from day 5 cultures. For continued culture, after 7–10 days culture and expansion in the presence of IL-2, CD8+ cells were restimulated at 7–10-day intervals. Cells (1 × 106 per ml) were restimulated with 3 × 104 irradiated (3,000 rads) B6 spleen cells previously pulsed with 5 μg ml−1 OVA for 30 min.

In the chimera expressing the OT-I TCR with or without CD8z and CD8b were made by retroviral transfection of TCR-deficient 58Z β − cells49. Chimeric genes encoding CD8β-YFP, CD8β-mCherry, CD3ζ-FP21, CD3ζ-eGFP, and Lck-eGFP, Lck-mCherry and CD95-Lck-Cerulean were constructed, inserted into retroviral vector pBMIN-Z (S. Kinoshita and G. Nolan, www.stanford.edu/group/nolan) and were expressed in Plat-E packaging cells. All the mutants were made using the Quickchange Site Mutagenesis Kit (Stratagene). Supercryptic of these mutants were used to transduce OT-I hybridsoma and OT-I CTL. For optimal FRET sensitivity and to avoid false-negative results, the acceptor (YFP or mCherry) should be in a stoichiometric ratio to the donor (eGFP or eYFP). Changes in the non-optimal acceptor/donor molar ratio for FRET; that is, an acceptor/donor molar ratio of 1:1 to 3:1. mCherry-sb2(ZAP70)30 (Addgene plasmid 27137) was cloned into pBMN-Z vector using Hind-III and Not-I sites. Two different sequences were used for Lck knockdown in pGFP-RS-U6 small hairpin RNA vector (#2323ctggcaagacaacctggtta and #43gagaacattgacgtgtgtg). OT-I hybridsoma cells were maintained in IMDM medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml−1 penicillin/streptomycin, 50 mM β-mercaptoethanol, 500 μM ml−1 G418 (selecting for TCRz) and 3 μg ml−1 puromycin (selecting for TCRβ).

FRET microscopy. A dual-camera system specifically designed for FRET imaging was used for imaging, allowing simultaneous acquisition of donor emission and acceptor emission during donor excitation and fast changes between donor and acceptor excitation. This consisted of two CoolSnapHQ cameras (Roper) attached to a Zeiss 200M microscope through a beam splitter (custom 520LPXR; Chroma) and stationary emission filters. A DG4 galvo illuminator customized with a 300-W Xenon lamp (Sutter) was used for rapid wavelength switching. The system was run by Slidebook 6.0 software (3I). The optical filters were as follows (center/bandpass): YFP excitation, 504/12–25 nm; YFP emission, 542/27 nm; CFP excitation, 427/10 nm; CFP emission, 472/30 nm; mCherry excitation, 589/15 nm; mCherry emission, 633/22 nm; Cy5 excitation, 620/52 nm; Cy5 emission, 675/50 nm. Bleed-through of one channel to the other (Chroma) was recorded as 0.2–0.5% with 2 × 2 binning and a 10, 1.4 numerical aperture objective, and soft flatfield correction was used. Live cells were imaged in Hank’s balanced salt solution (HBSS) medium supplemented with 1 mM CaCl2, 1 mM MgCl2 and 10 mM HEPES and without antibiotics and were maintained at 37 °C by the FCS2 laser head in a humid chamber (Biotoptechs). Pictures were acquired with a four-laser (405, 488, 561 and 647 nm) motorized TIRF module equipped with a laser (405, 488, 561 and 647 nm) motorized TIRF module was used and images were obtained in a Hamamatsu ORCA Flash 4.0 camera.

TIRF microscopy. TIRF microscopy experiments were performed using a × 100 1.45 numerical aperture TIRF objective (Nikon) on a Nikon TE2000U microscope custom modified with a TIRF illumination module. Laser illumination (488 and 561 nm lasers) were adjusted to impinge on the coverslip at an angle so as to yield a calculated evanescent field depth (d) < 100 nm. Images were acquired on a 14-bit, cooled charge-coupled device camera (CoolSnap HQ2, Roper) controlled through NIS-Elements software (Nikon Inc.). The images were recorded using 300–500 ms exposures depending on the fluorescence intensity of the sample. In some experiments, an Olympus IX81 microscope equipped with a four-laser (405, 488, 561 and 647 nm) motorized TIRF module was used and images were obtained in a Hamamatsu ORCA Flash 4.0 camera.

FRET analysis. Crosstalk compensation and extraction of donor-normalized FRET was performed using a three-filter set algorithm as described previously21,30,50. For each focal position, three exposures were registered: Ia (donor excitation/donor emission), Ia (acceptor excitation/acceptor) and I (donor excitation/acceptor, or more generally DA). Background was subtracted based on local fluorescence averaged from a user-specified, cell-free region of each image. The bleed-through coefficients of donor into the image and acceptor into the DA image were calibrated using T cells transfected with either CD8z-donor or CD8b-acceptor alone. Acceptor > DA bleed-through was a 12% for eYFP in the dual-camera system and a 15% for mCherry in the TIRF system, donor > DA bleed-through was 54% for eCFP in the dual-camera system and 10.6% for eGFP in the TIRF system. Sensitized emission was calculated by simply correcting the DA image for donor and acceptor bleed-through: I = Ia − 2IaG − dIaG. To get photobleaching correction (Ei), we used the following formula: Ei = Ia/I = 1 − (1 − Ei)G, where G is the independently calibrated ratio of sensitized emission in the Ia filter set after photobleaching, to donor recovery in the Ia filter set after acceptor photobleaching. For eCFP and eYFP in the dual-camera system G = 3.83 ± 0.1, for eCFP and mCherry in the TIRF system G = 2.1 ± 0.1. We have implemented this FRET method as an easy-to-use plugin ImageJ macro (from Roszk et al.31). The FRET image was masked to accept only regions in which donor intensity was > 4 times above background noise. Cells with donor/acceptor ratios outside the stoichiometric range of 1:1 to 3:1, as well as movement artefacts, were excluded from analysis. The average FRET was calculated from the synapse.

Ca2+ flux imaging. H2-Kb-OVA-expressing CHO cells were plated in poly-l-lysine-coated chambers then, after washing in Ca2+1/25 mmol/l free medium containing 5% (v/v) FCS, 100 U ml−1 penicillin, 10 mg ml−1 streptomycin, 1 mM EGTA, OT-I T-cell hybridsoma cells expressing both CD8z and CD8b loaded with 2 μM Ca2+ sensitive dye Fluo-4 were added on top. Imaging of Fluo-4 fluorescence was conducted in HBSS supplemented with 2.5 mM CaCl2,

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Cell surface molecular density and Adhesion Frequency Assay. For every micropipette experiment, site densities of MHCp on RBCs and TCR and CD8 on T cells were measured by flow cytometry. To measure the MHCp site density, RBCs were incubated with PE-conjugated monoclonal Ab 3H2627 (for H-2K^b^) at 10 μg/ml in 200 μl of FACS buffer (RPFI 1640, 5 mM EDTA, 1% BSA and 0.02% sodium azide) on ice for 40 min. Similarly, CD8αα subunit (clone 53-6-7), CD8β subunit (clone H35-17.2), TCR Vα2 (clone B20.1) and CD3ε (clone 145-2-C11) expressed on T cells were stained with their respective PE-conjugated monoclonal Abs (1:500 dilution, ebioscience). CD8 is expressed as either an α/α homodimer or an α/β heterodimer. Therefore, it is assumed that the site density of CD8β equals that of CD8αα, whereas the site density of CD8βεα equals half of the site-density difference between CD8β and CD8αα.

For the adhesion frequency assay, an OT-1 T cell and a RBC were aspirated by respective pipettes and driven in and out of contact with controlled area and duration. Adhesion was observed from stretching of the RBC on T-cell retraction. This contact-retraction cycle was either repeated 50 times for a given contact duration on each cell pair, and 3–5 cell pairs were used to estimate an adhesion frequency P (mean ± s.e.m.). P αβ can be converted to the average number <n> by

\[
<n> = 1 - \frac{1}{P} \quad \text{and} \quad \text{ligand density} \quad m_{\text{sample}} = \text{to obtain normalized adhesion bonds} \quad <n>/m_{\text{sample}}.\]

Experiments were performed at room temperature.

Liposomes and lipid bilayer preparation. 1,2-dioleoyl-sn-glycerol-3-phosphocholine was mixed in chloroform with 0.2 mol% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-Cap-Biotin (Cap-biotin-PE), all the lipids were from Avanti Polar Lipids, dried under vacuum and sonicated in 2% OG. The liposome solution was then extruded through a 0.2-μm filter and dialysed against 41 of Tris saline buffer (Tris-HCl 20 mM, NaCl 50 mM). Glass slides (No. 1, 24 mm × 50 mm German borosilicate; Menzel-Gläser) were treated with Piranha solution (70% sulfuric acid and 30% hydrogen peroxide) following agonist and antagonist stimulation. Immunity 17, 809–822 (2002).

Kim, P. W., Sun, Z.-Y. J., Blacklow, S. C., Wagner, G. & Eck, M. J. A zinc clasp structure tethers Lck to T cell coreceptors CD4 and CD8. Science 301, 1725–1728 (2003).

Nika, K. et al. Constitutively active Lck kinase in T cells drives antigen signal transduction. Immunity 32, 766–777 (2010).

Boggon, T. J. & Eck, M. J. Structure and regulation of Src family kinases. Oncogene 23, 7917–7928 (2004).

Ehrlich, L. I. R., Ebert, P. J. R., Krummel, M. F., Weiss, A. & Davis, M. M. Dynamics of p56lkx translocation to the T cell immunological synapse following agonist and antagonist stimulation. Immunity 17, 809–822 (2002).

The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation. Immol. Rev. 191, 107–118 (2009).

Fooksman, D. R. et al. Functional anatomy of T cell activation and synapse formation. Annu. Rev. Immunol. 28, 79–105 (2010).

Huse, M. et al. Spatial and temporal dynamics of T cell receptor signaling with a photoactivatable agonist. Immunity 27, 76–88 (2007).

Jiang, N. et al. Two-stage cooperative T cell receptor–peptide major histocompatibility complex-CD8 trimolecular interactions amplify antigen discrimination. Immunity 12, 23–33 (2000).

Holler, P. D. & Kranz, D. M. Quantitative analysis of the contribution of TCR/pemMHC affinity and CD8 to T cell activation. Immunity 18, 255–264 (2003).

Wooldridge, L. et al. Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface. J. Immunol. 170, 2794–27951 (2005).

Boggon, T. J. & Eck, M. J. Structure and regulation of Src family kinases. Oncogene 23, 7917–7928 (2004).

van der Merwe, P. A. & Cordoba, S. P. Late arrival: recruiting coreceptors to the T cell receptor complex. Immunity 34, 1–3 (2011).

Xu, H. & Littman, D. R. A kinase-independent function of Lck in potentiating antigen-specific T cell activation. Cell 74, 633–643 (1993).

Lee-Fruman, K. L., T. & Burakoff, S. J. Role of the Lck Src homology 2 and 3 domains in protein tyrosine phosphorylation. J. Biol. Chem. 271, 25003–25010 (1996).

Fragoso, R. C., Pyarajan, S., Irie, H. Y. & Burakoff, S. J. A CDLk/Dck transgene is able to drive thymocyte differentiation. Immunity 177, 6007–6017 (2006).

Zal, T., Zal, M. A. & Gascoigne, N. R. J. Inhibition of T cell receptor-coreceptor interactions by antigen ligand. Nat. Immunol. 3, 2794–27951 (2002).

Zal, T., Zal, M. A. & Gascoigne, N. R. J. The immunological synapse: a molecular machine controlling microbial infection. Nat. Immunol. 7, 759–792 (2006).

Gascoigne, N. R. J. & Zal, T. Molecular interactions at the T cell antigen-presenting cell interface. Curr. Opin. Immunol. 16, 114–119 (2004).

Yachi, P. P., Ampudia, J., Gascoigne, N. R. J. & Zal, T. Nonstimulatory peptides contribute to antigen-induced CD8αα T-cell receptor interaction at the immunological synapse. Nat. Immunol. 6, 785–792 (2005).

Yachi, P. P., Ampudia, J., Zal, T. & Gascoigne, N. R. J. Altered peptide ligands induce delayed CD8-T cell receptor interaction—a role for CD8 in distinguishing antigen quality. Immunity 25, 203–211 (2006).

Mallal, M. et al. The T cell receptor’s alpha-chain connecting peptide motif promotes close approximation of the CD8 coreceptor allowing efficient signal transduction. Immunity 180, 8211–8221 (2021).

Grakoui, A. et al. The immunological synapse: a molecular machine controlling T cell activation. Science 285, 221–227 (1999).

Hoeter, J. A. H. et al. Coreceptor affinity for MHC defines peptide specificity requirements for TCR interaction with coactivator peptide-MHC. J. Exp. Med. 210, 1807–1821 (2013).

Rybakin, V., Clamme, J.-P., Ampudia, J., Yachi, P. P. & Gascoigne, N. R. J. CD8αα and -ββ isoforms are equally recruited to the immunological synapse through their ability to bind to MHC class I. EMBO Rep. 12, 1251–1256 (2011).

Forster, T. Energy migration and fluorescence. 1946. J. Biomed. Opt. 17, 011002 (2012).

Zal, T. & Gascoigne, N. R. J. Photobleaching-corrected FRET efficiency imaging of live cells. Biophys. J. 86, 3923–3939 (2004).

Groves, J. T. & Dustin, M. L. Supported planar bilayers in studies on immune cell adhesion and communication. J. Immunol. Methods 278, 19–32 (2003).

Devine, L. et al. Mapping the binding site on CD8 beta for MHC class I reveals mutants with enhanced binding. J. Immunol. 177, 3930–3938 (2006).

Gascoigne, N. R. J. et al. Inability of CD8 alpha’ polypeptides to associate with p56lk and -ββ isoforms are equally recruited to the immunological synapse through their ability to bind to MHC class I. EMBO Rep. 12, 1251–1256 (2011).

Yudushkin, I. A. & Vale, R. D. Imaging T cell receptor activation reveals accumulation of tyrosine-phosphorylated CD8αα in the endosomal compartment. Proc. Natl Acad. Sci. USA 107, 22128–22133 (2010).
37. Iwashima, M., Irving, B. A., van Oers, N. S., Chan, A. C. & Weiss, A. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science* **263**, 1136–1139 (1994).
38. van Oers, N. S., Killeen, N. & Weiss, A. Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. *J. Exp. Med.* **183**, 1053–1062 (1996).
39. Au-Yeung, B. B. *et al.* The structure, regulation, and function of ZAP-70. *Immunol. Rev.* **228**, 41–57 (2009).
40. Palacios, E. H. & Weiss, A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene* **23**, 7990–8000 (2004).
41. Legname, G. *et al.* Inducible expression of a p56lck transgene reveals a central role for Lck in the differentiation of CD4 SP thymocytes. *Immunity* **12**, 537–546 (2000).
42. Seddon, B. Long-term survival but impaired homeostatic proliferation of naive T cells in the absence of p56lck. *Science* **290**, 127–131 (2000).
43. Levin, S. D., Abraham, K. M., Anderson, S. J., Forbush, K. A. & Perlmutter, R. M. The protein tyrosine kinase p56lck regulates thymocyte development independently of its interaction with CD4 and CD8 coreceptors [corrected]. *J. Exp. Med.* **178**, 245–255 (1993).
44. Yamaguchi, H. & Hendrickson, W. A. Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation. *Nature* **384**, 484–489 (1996).
45. Paster, W. *et al.* Genetically encoded Förster resonance energy transfer sensors for the conformation of the Src family kinase Lck. *J. Immunol.* **182**, 2160–2167 (2009).
46. Stirnweiss, A. *et al.* T cell activation results in conformational changes in the Src family kinase Lck to induce its activation. *Sci. Signal.* **6**, ra13 (2013).
47. Thome, M., Duplay, P., Guttinger, M. & Acuto, O. Syk and ZAP-70 mediate recruitment of p56lck/CD4 to the activated T cell receptor/CD3/zeta complex. *J. Exp. Med.* **181**, 1997–2006 (1995).
48. Thome, M., Germain, V., DiSanto, J. P. & Acuto, O. The p56lck SH2 domain mediates recruitment of CD8/p56lck to the activated T cell receptor/CD3/zeta complex. *Eur. J. Immunol.* **26**, 2093–2100 (1996).
49. Stotz, S. H., Bolliger, L., Carbone, F. R. & Palmer, E. T cell receptor (TCR) antagonism without a negative signal: evidence from T cell hybridomas expressing two independent TCRs. *J. Exp. Med.* **189**, 253–264 (1999).
50. Zal, T. & Gascoigne, N. R. J. Using live FRET imaging to reveal early protein-protein interactions during T cell activation. *Curr. Opin. Immunol.* **16**, 418–427 (2004).
51. Roszik, J., Lisboa, D., Szőllősi, J. & Vereb, G. Evaluation of intensity-based ratiometric FRET in image cytometry--approaches and a software solution. *Cytometry A* **75**, 761–767 (2009).

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**Author contributions**
J.C. performed most of the experiments. J.B., J.A.H.H., G.F., Q.W., R.Z. and J.A. generated antigen-presenting cell lines, constructs and mutants. V.I.Z. and J.-s.H. performed adhesion frequency assays. J.C., J.B., V.I.Z. and J.-s.H. analysed data. J.C. and N.R.J.G. designed the project with help and insight from J.C., J.B. and C.Z. J.C. and N.R.J.G. wrote the manuscript.

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