Biophysical mechanism of T-cell receptor triggering in a reconstituted system

John R. James1 & Ronald D. Vale1

A T-cell-mediated immune response is initiated by the T-cell receptor (TCR) interacting with peptide-bound major histocompatibility complex (pMHC) on an infected cell. The mechanism by which this interaction triggers intracellular phosphorylation of the TCR, which lacks a kinase domain, remains poorly understood. Here, we have introduced the TCR and associated signalling molecules into a non-immune cell and reconstituted ligand-specific signalling when these cells are conjugated with antigen-presenting cells. We show that signalling requires the differential segregation of a phosphatase and kinase in the plasma membrane. An artificial, chemically controlled receptor system generates the same effect as TCR-pMHC, demonstrating that the binding energy of an extracellular protein–protein interaction can drive the spatial segregation of membrane proteins without a transmembrane conformational change. This general mechanism may extend to other receptors that rely on extrinsic kinases, including, as we demonstrate, chimaeric antigen receptors being developed for cancer immunotherapy.

In addition to intercellular communication mediated by soluble molecules, two cells can transmit signals through membrane-associated receptors and ligands. Adaptive immunity represents such a system, in which the MHC protein on the surface of antigen-presenting cells (APCs) interacts with the TCR on T lymphocytes. If the TCR binds pMHC of the right complementarity, the interaction results in tyrosine phosphorylation of the TCR (herein referred to as TCR 'triggering') and the initiation of signals that activate the T cell1. The TCR has no intrinsic kinase activity, unlike many other receptors2, and instead relies on a T-cell-specific kinase called Lck (ref. 3). Also distinct from other systems, the phosphorylatable tyrosine residues of the TCR (the immunoreceptor tyrosine-based activation motifs (ITAMs))4 do not reside on the polypeptides that contact the pMHC and instead are contained on tightly associated CD3 subunits (ζ, δ, εζ, εζζ). The phosphorylated ITAMs then bind a second kinase, ZAP70, which is subsequently activated and drives downstream signalling5.

Despite considerable work, the mechanism by which pMHC binding leads to TCR triggering remains poorly understood (reviewed in ref. 6). Some models propose that pMHC binding evokes a conformational change in the TCR that makes its cytoplasmic ITAM domains more accessible to Lck kinase6. Alternative triggering hypotheses include activation through the aggregation of TCR molecules6, and 'kinetic segregation', where TCR phosphorylation is favoured by its partitioning into plasma membrane domains that contain Lck kinase but are depleted of CD45 (also known as PTPRC), an abundant transmembrane phosphatase. However, although TCR clustering6 and the segregation of CD45 away from the TCR have been observed7, it has not been established whether such events are necessary or sufficient for signal transduction across the plasma membrane. In addition, the physical basis of protein segregation within the plasma membrane is unclear.

Reconstitution of a biological phenomenon with defined components has proven to be a powerful means for dissecting molecular mechanisms. We have made use of this approach by introducing the genes encoding the TCR and other proteins required for regulating its phosphorylation into a non-immune cell and recapitulating TCR triggering when this cell forms a conjugate with an APC. Because each protein can be introduced separately and is genetically engineered, this system has allowed us to test models of TCR triggering and the roles of individual proteins in a manner that is difficult to achieve with native T cells.

Reconstitution of regulated TCR triggering

We first sought to reconstitute Lck-mediated TCR phosphorylation in a non-immune cell and then determine which factors are needed to keep the TCR quiescent (Fig. 1a). As the basis of our reconstitution, we expressed11 the complete set of protein chains of the 1G4 TCR (ref. 12) in the plasma membrane of HEK cells (hereafter referred to as HEK-1G4) (Supplementary Methods and Supplementary Fig. 1). The expressed TCR complex did not show detectable phosphorylation (assayed by a phospho-specific antibody to the CD3 ε subunit) unless Lck and ZAP70 were co-expressed (Fig. 1b). Lck kinase activity, as detected by measuring levels of activating (Ytr 394) and inhibitory (Ytr 505) phosphorylation7, seemed to be unaffected by the presence of the TCR or ZAP70 (Fig. 1b). However, ZAP70 activity, as measured by increased Tyr 493 phosphorylation7, was only detectable in the presence of both Lck and the TCR (Fig. 1b), which is in agreement with previous data suggesting that this kinase is inactive until it binds to phosphorylated CD3ζ ITAMs8 (Fig. 1a). We confirmed the activity of ZAP70 by demonstrating phosphorylation of co-expressed LAT, its downstream substrate and critical adaptor protein for T-cell signalling (Supplementary Fig. 2a).

To establish a quiescent system that could be activated by pMHC, we next sought to restrain the kinase activity of Lck. CSK induces an 'inactive' conformation of Lck by phosphorylating its carboxy terminus (Ytr 505) (ref. 16). However, co-expressing CSK and CSK-binding protein (GBP, also known as PAG1), which localizes CSK to the plasma membrane (Fig. 1a), was insufficient to repress Lck phosphorylation of CD3ζ (Fig. 1c). CD45 is a tyrosine phosphatase that modulates T-cell signalling in a complex manner by dephosphorylating the inhibitory Tyr 505 and activating Tyr 394 of Lck (refs 17, 18) and the ITAM tyrosines of the TCR (Fig. 1a). Co-expression of CD45 with Lck severely

1Howard Hughes Medical Institute and Department of Cellular and Molecular Pharmacology, University of California, San Francisco, 600 16th Street, San Francisco, California 94158, USA.

©2012 Macmillan Publishers Limited. All rights reserved
with a short peptide (ESO9V) (ref. 19) and expressed in the Raji B-cell line as the APC (Fig. 2a and Supplementary Methods). Because HEKs and Raji B cells have no intrinsic affinity for each other (Supplementary Fig. 3a), we expressed the immune-cell adhesion proteins CD2 and ICAM1 on the HEK-1G4 cells (Fig. 2a), which can interact with their respective counter-receptors CD58 and LFA-1 expressed endogenously on Raji cells (Supplementary Fig. 3a). This strongly increased the conjugation between the two cell types, with the LFA-1–ICAM1 interaction being the predominant driver (Supplementary Fig. 3a).

**Reconstitution of a T cell–APC conjugate**

Having identified a minimal set of components that could be expressed in HEK-1G4 cells to mimic the basal or ‘off’ state of their T-cell counterpart, we next attempted to trigger signalling by having the 1G4 TCR interact with its ligand, the MHC class-I complex bound to a short peptide (ESO9V) and expressed in the Raji B-cell line as an APC (Fig. 2a). We have used the TCR CD45+CD45Int, which are not activated under these conditions (Supplementary Fig. 1). With the use of CD58 and LFA-1, we were able to form conjugates between Raji cells and HEK-1G4 cells expressing the components shown in Supplementary Fig. 3a. Thus, we expressed the immune-cell adhesion proteins CD2 and ICAM1 on the HEK-1G4 cells (Fig. 2a), which can interact with their respective counter-receptors CD58 and LFA-1 expressed endogenously on Raji cells (Supplementary Fig. 3a). This strongly increased the conjugation between the two cell types, with the LFA-1–ICAM1 interaction being the predominant driver (Supplementary Fig. 3a).

**Figure 1** | Regulatable TCR triggering in an engineered HEK cell line.

a, Schematic representation of molecules transfected into HEK cells. b, Western blot of phosphorylated proteins after transfection of HEK cells with selected molecules (green circles). c, Cells transfected with the TCR, Lck and ZAP70 were transfected with additional molecules (green circles), showing the synergistic action of modulatory proteins CSK, CBP and CD45 to restrain Lck. The decreased phosphorylation between lanes five and seven is due to endogenous CSK recruitment. d, Confocal images of HEK-1G4 cells (expressing components in lane eight of Supplementary Fig. 3) showed the synergistic action of modulatory proteins CSK, CBP and CD45 on ZAP70–GFP accumulation at the plasma membrane (Fig. 1d, e), whereas co-expression of CSK, CBP and CD45 in HEK-1G4 cells caused ZAP70–GFP to accumulate at the plasma membrane (Fig. 1d, e). e, Quantification of ZAP70 relocalization with indicated molecules transfected in HEK-1G4 cells. Data are mean ± s.e.m. of three independent experiments (n = 300 cells per experiment). Scale bars, 5 μm.

**Figure 2** | The exclusion of CD45 phosphatase is necessary and sufficient for TCR triggering.

a, Proteins expressed in HEK-1G4 for cell conjugation. b, HEK-1G4 cells, expressing all components shown in a, were conjugated with APCs (Raji cells) expressing cognate pMHC (ESO9V) or a control pMHC. Coloured boxes denote protein representation in the overlay image. c, A representative line profile of membrane fluorescence from CD45 (red), pMHC (blue) and ZAP70 (green) at the conjugate interface. d, Quantification of triggering (defined as unambiguous recruitment of ZAP70 to conjugate region) for all conjugates described in text. Data are mean ± s.e.m. of independent experiments (n = 4 or 5, 30–150 conjugates per experiment). e, Forcing CD45 into the conjugate region by fusing to CD2 (CD2exCD45Int) blocks TCR triggering (ZAP70 remains cytosolic). Quantification is shown in d. Scale bars, 5 μm.
We found that the HEK-1G4 cells formed a cup-shaped contact around the APC expressing antigenic pMHC (Supplementary Movie 2), which was notably similar to T cell–APC conjugates. pMHC was concentrated at the site of cell–cell interaction (Fig. 2b, c), and importantly, ZAP70 in the HEK-1G4 cells translocated to the plasma membrane in this region, showing that TCR triggering had occurred (Fig. 2b–d; confirmed by immunoblotting in Supplementary Fig. 2b). Triggering was rapid and could be observed within 1 min of cell contact (Supplementary Movie 3). Membrane translocation of ZAP70 did not occur when HEK-1G4 cells were conjugated with Raji cells expressing a control peptide–MHC complex (Fig. 2b, d) or in HEK-1G4 cells lacking transfectect Lck (Fig. 2d and Supplementary Fig. 2b). We also found that the ITAMs of CD3ζ were sufficient for ligand-specific ZAP70 translocation by using a minimal TCR complex with the intracellular sequences of CD3γ, δ and ε truncated after the transmembrane domain (TCRExInt, where ‘Ex’ denotes the extracellular and transmembrane parts of the molecule and ‘Int’ denotes the intracellular part; Fig. 2d). Although the APC used in these experiments expressed the cognate pMHC at high levels (~50 pMHC per μm²), ZAP70 recruitment was still observable at lower, more physiological, levels of antigenic pMHC (~3 pMHC per μm²) (refs 21, 22) (Supplementary Figs 1 and 4). In summary, an APC expressing the appropriate pMHC is able to elicit a specific TCR-triggering response from our reconstituted HEK-1G4 cell.

Disrupting the actin cytoskeleton severely inhibits T-cell activation, but its role in TCR triggering is uncertain. Depolymerization of actin filaments in HEK-1G4 cells before mixing with APCs significantly decreased the number of conjugates (Supplementary Fig. 3b), but cells that did interact still showed ZAP70 recruitment (Supplementary Fig. 3c). This result indicates that the cytoskeleton facilitates the initial cell–cell interactions but is not essential for TCR triggering in this reconstituted cell system.

Mechanistic insight into how the TCR was triggered by pMHC came from the observation that CD45 phosphatase was excluded from the cell–cell interface where the TCR was bound to its cognate ligand (Fig. 2b, c), whereas Lck remained included (Supplementary Fig. 5a). As complete repression of Lck phosphorylation of the TCR required CD45, CSK and CBP (Fig. 1c), the segregation of CD45 away from the TCR would be predicted to shift the steady-state balance towards TCR phosphorylation. Previous studies have also found that most CD45 molecules are excluded from the interface of real T cell–APC conjugates and could be involved in triggering. However, it has been difficult to ascertain whether CD45 exclusion is responsible for, or a consequence of, TCR triggering. To show more definitively that CD45 exclusion has a causal role, we redirected phosphatase activity into the conjugate interface, respectively, in the absence of rapamycin. However, ZAP70 was recruited to the membrane in the zones of CD45 exclusion only when the separated components of the TCR (TCRExFKBPInt and FRB–CD3ζInt) were brought together with rapamycin (Fig. 3b, c). Time-lapse imaging showed no discernible delay between the translocation of FRB–CD3ζInt to the plasma membrane and the subsequent recruitment of ZAP70 (Fig. 3d and Supplementary Movie 4).

The mechanism of CD45 segregation

We next wished to explore what forces produce CD45 exclusion. MHC bound with a control peptide did not elicit CD45 segregation (Fig. 2b), therefore the adhesion pairs (CD2–CD58 and LFA-1–ICAM1) must be incapable of driving exclusion (Supplementary Fig. 5c). We next tested the TCR–pMHC interaction alone, and found that it was sufficient for conjugation (Supplementary Fig. 3a) and CD45 segregation (Fig. 4a). Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) experiments showed that the TCR remained mobile after pMHC binding and free to escape the conjugated region, showing that the segregated zone was not formed by an immobile aggregate and remained contiguous with the rest of the cell surface (Supplementary Fig. 6). Thus, the TCR–pMHC interaction
Data are mean ± s.e.m. (n = 20) for each construct.

is necessary and sufficient for CD45 exclusion, with no requirement for downstream TCR triggering/signalling.

Next, we examined how the TCR–pMHC interaction affected the spacing of the two plasma membranes of the interacting cells. Using a subdiffraction-resolution method, we measured the separation between a GFP-tagged TCR in the HEK cells and mCherry–pMHC in the APC (Fig. 4b and Supplementary Methods). The measured distance of ~31 nm (Fig. 4b) suggests a cell–cell separation of ~15 nm, which agrees well with the 13 nm cell-to-cell distance between T cells and conjugated APCs measured by electron microscopy (ref. 28). By contrast, the membrane separation for Raji cells expressing control pMHC conjugated through adhesion molecules alone (ICAM1–LFA-1 or ICAM1–LFA-1 and CD2–CD58) was much greater (Fig. 4b). These results show that the TCR–pMHC interaction brings the membranes much closer together than occurs with the adhesion molecules.

Proteins with extended extracellular domains (such as CD45) might be prevented from entering regions of close membrane apposition, as suggested by the kinetic-segregation model⁸. We tested this hypothesis by fusing the intracellular phosphatase domains of CD45 to an extracellular domain (from CD86) of comparable size to the TCR (termed CD86ExCD45Int). CD86ExCD45Int was also excluded from the cell–cell interface, although its exclusion (Fig. 4c) and TCR triggering (Supplementary Fig. 7a, b) were somewhat lower than that seen with the large CD45 construct (Fig. 4c). Because of this unanticipated exclusion of a protein with a small extracellular domain, we next tested mCherry fusion proteins of a series of membrane proteins with different properties: CD58, a transmembrane protein with an equivalently sized extracellular domain to CD86; β2-adrenergic receptor (β2AR, also known as ADRB2), a seven-transmembrane protein with small extracellular loops; and a prenylated version of the fluorophore linked to the inner leaflet of the bilayer by using a short targeting sequence (CAAX) (Fig. 4c). CD58 was excluded to a similar level as CD86ExCD45Int, showing that the segregation of the latter construct was not due to the intracellular phosphatase domains of CD45 (Fig. 4c). β2AR, a protein with a large lateral footprint, was also partially excluded (Fig. 4c), possibly through a crowding effect arising from the high density of pMHC–TCR in the conjugate region. However, the prenylated mCherry was distributed evenly throughout the cell membrane, showing no exclusion by TCR–pMHC (Fig. 4c).

The above experiments cannot explain why CD2 (Supplementary Fig. 5b) and CD2ExCD45Int (Fig. 2e), which has extracellular domains similar in size to CD86, were not excluded from the cell–cell contact zone. We speculated that binding of CD2 to its ligand (CD58) expressed endogenously on the APC provided a counteracting force to constrain CD2 within the conjugate region. If this were true, then expressing CD28, the binding partner for CD86, on the APC should diminish the TCR–pMHC-mediated segregation of CD86ExCD45Int. Indeed, when CD86ExCD45Int-expressing HEK-1G4 cells were conjugated with APCs co-expressing CD28, CD86ExCD45Int localized at the cell–cell interface and TCR triggering was greatly decreased (Supplementary Fig. 7b, c).

In summary, the TCR–pMHC interaction alone is capable of excluding plasma membrane proteins with extracellular extensions and that exclusion can be overcome by the energy provided by binding to a protein partner on the APC.

Triggering of artificial receptors

The preceding experiments suggested that TCR triggering results from CD45 segregation, which is driven by the binding interaction between the TCR and pMHC. If this is true, then interactions between extracellular domains of membrane proteins with the proper spacing and affinity might elicit comparable effects to TCR–pMHC, as has been shown in T cells²⁸. To explore this idea, we engineered a chemically controlled, cell-surface-receptor system consisting of a transmembrane protein with extracellular FKBP and the intracellular CD3ζ ITAM domains expressed in the HEK cell (FKBPExInt, mimicking the TCR) and a transmembrane protein with an extracellular FRB expressed in the APC (mimicking the pMHC) (Fig. 5a). FKBPExInt and FRBEx will only interact in the presence of rapamycin, forming a complex that spans a similar distance to TCR–pMHC. In the absence of rapamycin, conjugates formed (through the LFA-1–ICAM1 interaction) but ZAP70 was not recruited to the membrane (Fig. 5b, c). However, with rapamycin, FKBPExInt receptor clusters were observed at the cell–cell interface, even with low levels of FRBEx ligand that are equivalent to physiological densities of antigen pMHC (~5 molecules per μm²; Supplementary Fig. 1). Furthermore, CD45 was excluded from and ZAP70 was recruited to these receptor clusters, indicating that triggering had occurred (Fig. 5b–d).

Our artificial receptor is structurally analogous to chimeric antigen receptors (CARs), which have an extracellular single-chain antibody fragment fused to a transmembrane sequence and cytoplasmic CD3ζ ITAMs. When the CD19-specific CAR (ref. 29), which has

©2012 Macmillan Publishers Limited. All rights reserved
shown promise in recent clinical trials as a gene therapy treatment of leukaemia, was expressed in our reconstituted HEK cells and these were conjugated with Raji B cells (which are CD19⁺), we observed CAR clustering, ZAP70 recruitment and CD45 exclusion (Fig. 5e, f). This triggering was ligand specific, as conjugation with CD19⁻ cells did not produce these effects (Fig. 5e, f). Interestingly, the interface of many CD19⁻ CAR HEK cell and CD19⁺ Raji B-cell conjugates showed a highly convoluted membrane surface (Supplementary Movie 5), which was not seen for cells interacting through either TCR–pMHC or FKBP¹⁺⁻FRB¹⁻. This membrane effect could be a product of high-affinity antibody binding and might warrant further investigation for its relevance to CAR potency or potential side effects.

**Conclusion**

Our reconstitution experiments show a physical mechanism for TCR triggering that differs from dimerization or conformational-change models proposed for many cell-surface receptors. We find that the binding energy of the TCR–pMHC interaction generates an exclusion force for membrane proteins with large and/or unligated extracellular domains, even in the absence of downstream signalling. By linking an inhibitory phosphatase activity to a transmembrane protein (CD45) that is subject to the exclusion force and an activating kinase (Lck) to the inner leaflet of the membrane that is not, the TCR–pMHC interaction can shift the kinase–phosphatase balance and thus trigger the TCR, as first suggested by the kinetic–segregation model. Because the FKBP–rapamycin–FRB or antibody–antigen modules can replace the extracellular TCR–pMHC interaction, conformational changes in the TCR are unlikely to be critical for the fundamental mechanism of triggering. Co-receptors and actin also do not seem to be essential for transducing TCR–pMHC binding across the plasma membrane, although it is very probable that they are necessary for achieving the high sensitivity and full selectivity of T-cell activation. This could be tested in the future by pushing our reconstituted system to respond to very low antigen densities using more sensitive read-outs of triggering.

The precise mechanism by which the TCR–pMHC interaction results in protein exclusion remains elusive, although our data provide certain clues. Exclusion according to the kinetic-segregation model is based on the greater size of the extracellular domain of CD45 compared to the TCR–pMHC complex. However, although size influences the extent of exclusion, our data show that membrane proteins with small extracellular domains are also excluded, suggesting that additional forces must be acting on the system. We speculate upon the driving force in the following model, which incorporates previous findings. Within the initial adhesion mediated by LFA-1–ICAM1 between the T cell and APC, transient fluctuations bring the two membranes in closer apposition, allowing TCR and pMHC molecules to interact (Fig. 6a). The binding energy of this interaction must be sufficient to overcome unfavourable membrane bending and compression of the large proteins that constitute the local membrane bending and may enhance the local exclusion of larger molecules by TCR–pMHC interactions. C. Consolidation of discrete contact regions serves to minimize unfavourable membrane bending and leads to the exclusion of smaller proteins that do not provide any counteracting ligand-binding free energy. See text for details.
glycocalyx, such as CD45, which occur when the two membranes regions are brought close together (Fig. 6b). In a critical next step, separate regions of close membrane apposition consolidate passively into larger, contiguous regions, resulting in a net decrease in membrane bending, as has been previously suggested.18 Proteins that do not provide any binding energy will be excluded over time, because, as they diffuse from the region, interacting molecules will be further clustered as the area of close apposition is minimized (Fig. 6c). These same principles may explain how TCR microdomains initiate when T cells interact with pMHC on a supported lipid bilayer.26–38 (note the small exclusion zones in our experiments at low ligand densities (Fig. 5d and Supplementary Fig. 4)).

Overall, our data suggest how the binding energy associated with specific molecular recognition events that take place between two interacting cells can be transduced into intracellular biochemical reactions that change cell behaviour. This model provides a plausible mechanism to explain how CARs trigger T cells to kill cancerous cells29 and may apply to other cell types that signal using membrane-bound receptors and ligands.

METHODS SUMMARY

Multiple proteins were expressed in HEK cells using a combination of transient and stable expression by lentiviral transduction; proteins were expressed at close to physiological levels and in the correct localization as described in Supplementary Methods. To create cell conjugates, the two cell types were centrifuged, resuspended at high density and placed on glass-bottomed dishes for live-cell imaging at 37 °C using spinning disc confocal microscopy. Image analysis, including the intermembrane distance algorithm, was performed using ImageJ and Matlab (Supplementary Methods). Full methods and supplementary material accompany this paper.

Received 14 November 2011; accepted 8 May 2012.

Published online 27 June 2012.

1. Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. T cell activation. Annu. Rev. Immunol. 27, 591–619 (2009).
2. Lemmon, M. A. & Schlessinger, J. Cell signaling by receptor tyrosine kinases. Cell 141, 1117–1134 (2010).
3. 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).
4. Love, P. E. & Hayes, S. M. ITAM mediated signaling by the T-cell antigen receptor. Oncogene 23, 7990–8000 (2004).
5. Xu, C. et al. Mechanisms for T cell receptor triggering. Nature Immunol. 11, 47–55 (2010).
6. van der Merwe, P. A. & Dushek, O. Mechanisms for T cell receptor triggering. Nature Immunol. 11, 47–55 (2010).
7. Lillemeier, B. F. et al. TCR and Lat are expressed on separate protein islands on T cell membranes and concentrate during activation. Nature Immunol. 11, 90–96 (2010).
8. Varma, R., Campi, G., Yokosuka, T., Saito, T. & Dustin, M. L. T cell receptor-proximal signals are sustained in peripheral microclusters and terminated in the central supramolecular activation cluster. Immunity 25, 117–127 (2006).
9. Szczypka, A. L. et al. Correction of multi-genic deficiency in vivo using a single ‘self–cleaving’ ZA peptide-based retroviral vector. Nature Biotechnol. 22, 589–594 (2004).
10. Aleksic, M. et al. Dependence of T cell antigen recognition on T cell receptor–peptide MHC confinement time. Immunity 32, 163–174 (2010).
11. Holst, J. et al. Scalable signaling mediated by T cell antigen receptor-CD3 ITAMs ensures effective negative selection and prevents autoimmunity. Nature Immunol. 9, 658–666 (2008).
12. Irving, B. A. & Weiss, A. The cytoplasmic domain of the T cell receptor ζ chain is sufficient to couple to receptor-associated signal transduction pathways. Cell 64, 891–901 (1991).
13. Deindl, S. et al. Structural basis for the inhibition of tyrosine kinase activity of ZAP-70. Cell 129, 735–746 (2007).
14. Bergman, M. et al. The human p50csk tyrosine kinase phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity. EMBO J. 11, 2919–2924 (1992).
15. Hermiston, M. L., Xu, Z. & Weiss, A. CD45: a critical regulator of signaling thresholds in immune cells. Annu. Rev. Immunol. 21, 107–137 (2003).
16. Saunders, A. E. & Johnson, P. Modulation of immune cell signaling by the leukocyte common tyrosine phosphatase, CD45. Cell. Signal. 22, 339–348 (2010).
17. Shen, J. L. et al. Structural and kinetic basis for heightened immunogenicity of T cell vaccines. J. Exp. Med. 201, 1243–1255 (2005).
18. Manz, B. N. Jackson, B. L., Petit, R. S., Dustin, M. L. & Groves, J. T cell triggering thresholds are modulated by the number of antigen within individual T cell receptor clusters. Proc. Natl Acad. Sci. USA 108, 9039–9054 (2011).
19. Vailutti, S., Dessing, M., Aktories, K., Gallati, H. & Lanzavecchia, A. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. J. Exp. Med. 181, 577–584 (1995).
20. Johnson, K. G., Bremley, S. K., Dustin, M. L. & Thomas, M. L. A supramolecular basis for CD45 tyrosine phosphatase regulation in sustained T cell activation. Proc. Natl Acad. Sci. USA 97, 10138–10143 (2000).
21. Leupin, O., Zaru, R., Larochette, T., Muller, S. & Vailutti, S. Exclusion of CD45 from the T cell receptor signaling area in antigen-stimulated T lymphocytes. Curr. Biol. 20, 277–280 (2010).
22. Irel, C. et al. CD45 ectodomain contribution interactions with GEMs and Lck activity for optimal TCR signaling. Nature Immunol. 4, 189–197 (2003).
23. He, X., Woodford-Thomas, T. A., Johnson, K. G., Shah, D. D. & Thomas, M. L. Targeting of CD45 protein tyrosine phosphatase activity to lipid microdomains on the T cell surface inhibits TCR signaling. Eur. J. Immunol. 32, 2578–2587 (2002).
24. Choudhury, K., Wiseman, D., Brown, M. H., Gould, K. & van der Merwe, P. A. T cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand. Nature 436, 578–582 (2005).
25. Porter, D. L., Levine, B. L., Katois, M., Biagg, A. & June, C. H. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N. Engl. J. Med. 365, 725–733 (2011).
26. Shaw, A. S. & Dustin, M. L. Making the T cell receptor go the distance: a topological view of T cell activation. Immunity 6, 361–369 (1997).
27. Qi, S. Y., Groves, J. T. & Chakraborty, A. K. Synaptic pattern formation during cellular recognition. Proc. Natl Acad. Sci. USA 98, 6548–6553 (2001).
28. Weik, T. R. & Lipowsky, R. Pattern formation during T-cell adhesion. Biophys. J. 87, 3665–3678 (2004).
29. Coombs, D., Dembo, M., Wofsy, C. & Goldstein, B. Equilibrium thermodynamics of cell–cell adhesion mediated by multiple ligand–receptor pairs. Biophys. J. 86, 1408–1423 (2004).
30. Alakoskela, J. et al. Mechanisms for size-dependent protein segregation at immune synapses assessed with molecular rulers. Biophys. J. 100, 2865–2874 (2011).
31. Burroughs, N. J. & Wulfing, C. Differential segregation in a cell–cell contact interface: the dynamics of the immunological synapse. Biophys. J. 83, 1784–1796 (2002).
32. Yokosuka, T. et al. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. Nature Immunol. 6, 1253–1262 (2005).
33. Campi, G., Varma, R., Dustin, M. L. Actin and agonist MHC–peptide complex-dependent T cell receptor microclusters as scaffolds for signaling. J. Exp. Med. 202, 1031–1036 (2005).
34. Bunnell, S. C. et al. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. J. Cell Biol. 158, 1263–1275 (2002).

 Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank: A. van der Merwe and Y. Cerundolo for the 1G4 TCR sequence. A. Weiss for cell lines and advice. C. June for the CD19 CAR construct. N. Stuurman and K. Thorn for microscopy help and members of the Vale laboratory for discussions. R.D.V. is a Howard Hughes Medical Institute investigator and J.R.J. is a fellow of the Jane Coffin Childs Memorial Fund.

Author Contributions J.R.J. conceived the study, collected the data and conducted the analyses. J.R.J. and R.D.V. designed the experiments and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to R.D.V. (vale@cmp.ucsf.edu).

©2012 Macmillan Publishers Limited. All rights reserved