Initiation of T cell signaling by CD45 segregation at ‘close contacts’

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It has been proposed that the local segregation of kinases and the tyrosine phosphatase CD45 underpins T cell antigen receptor (TCR) triggering, but how such segregation occurs and whether it can initiate signaling is unclear. Using structural and biophysical analysis, we show that the extracellular region of CD45 is rigid and extends beyond the distance spanned by TCR-ligand complexes, implying that sites of TCR-ligand engagement would sterically exclude CD45. We also show that the formation of ‘close contacts’, new structures characterized by spontaneous CD45 and kinase segregation at the submicron-scale, initiates signaling even when TCR ligands are absent. Our work reveals the structural basis for, and the potent signaling effects of, local CD45 and kinase segregation. TCR ligands have the potential to heighten signaling simply by holding receptors in close contacts.

The binding of foreign antigens presented by major histocompatibility complex proteins is known to result in TCR phosphorylation by the tyrosine kinase Lck, but how this occurs is not clear1. An important clue is that T cells express large amounts of CD45, a receptor-type protein tyrosine phosphatase (RPTP), on their surfaces2. Owing to its abundance (>100,000 molecules per cell and >3 molecules per TCR)3 and its high specific activity (10- to 1,000-fold higher than that of kinases)4, CD45 probably constrains tyrosine phosphorylation in resting cells. However, T cells lacking CD45 cannot be activated5, which suggests that it also has a role in the initiation of signaling. This is at least partly explained by findings that CD45 is required to activate Lck by dephosphorylating an inhibitory tyrosine at the C terminus of the kinase2.

A second important observation is that interventions acting globally to block phosphatases or activate kinases induce spontaneous T cell activation6–7. The kinetic-segregation (KS) model8,9 was proposed to explain how similar changes in kinase and/or phosphatase activity might be invoked. The KS model incorporated the observation10 that, owing to their large size, glycoalyx components such as CD45 are likely to be excluded from sites of TCR-ligand engagement. The KS model postulates that equilibrium between kinases and CD45 maintains low amounts of TCR phosphorylation until it is locally disturbed in favor of the kinases, which occurs when CD45 is sterically excluded from ‘close-contact zones’, hypothetical structures where TCRs and small proteins such as CD2 engage their ligands, resulting in net receptor phosphorylation13–19. However, segregation needs to occur over short lengths (probably <1 µm)11 to allow ligand discrimination.

Although it has substantial experimental support1,9, several key elements of the KS model are unverified. First, signaling-coincident formation of close-contact zones characterized by local CD45 and kinase segregation has not been reported. TCR microclusters exclude CD45 (ref. 12), but these structures are distinct from the close-contact zones predicted by the KS model to form TCR independently. Large-scale CD45 exclusion also occurs during immunological synapse formation13,14 but happens too late to affect early signaling15. Second, the mechanism of CD45-kinase segregation, if it occurs, is not clear, owing to uncertainty about the structural properties and dimensions of the extracellular region of CD45 (ref. 16). Third, it is unclear whether CD45 and kinase segregation could effect receptor triggering. CD45 blocks signaling when it can access TCR-ligand complexes17–19, but this confirms only that CD45-kinase segregation is required for signaling, not that it is sufficient. Fourth, as has been suggested20, CD45 exclusion could prevent rather than initiate signaling, because it is needed to activate Lck2. However, CD45 is notably unspecific21, and the TCR is even better substrate than Lck22, which suggests that CD45 exclusion could favor TCR phosphorylation. Finally, it is unknown whether receptor triggering relies on ligand engagement only or also on ligand-induced effects, such as receptor oligomerization or conformational changes1. However, the prediction that signaling could occur independently of ligand—if, for example, phosphatase-excluding close-contact zones were so large that kinase activity were effectively unopposed—offers a way to discriminate between the KS model and other, more ligand-centric explanations of receptor triggering.

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In this study we show that the extracellular domain (ECD) of CD45 is rigid and extends beyond the distance spanned by TCR-ligand complexes, which explains why it will be excluded from sites of TCR-ligand engagement. Live-cell imaging reveals the formation of submicron-scale structures that have several of the properties of close-contact zones postulated by the KS model. The formation of these structures, which we call close contacts, is capable of initiating signaling even when TCR ligands are absent. Together these findings identify the structural basis for, and the unexpectedly potent signaling effects of, local CD45-kinase segregation.

RESULTS
Structure of the modular region of the CD45 ECD
CD45 is expressed as multiple isoforms according to cell type, developmental stage and cell activation state. The CD45 ECD was predicted to comprise an N-terminal mucin-like region followed by a cysteine-rich domain (d1) and fibronectin type 3 (FN3) domains 2–4 (d2–d4). Human CD45 isoforms arise from alternative splicing of exons 4, 5 and 6 of the PTPRC gene, which alters the length of the mucin-like region. The smallest (CD45R0) and longest (CD45RABC) isoforms are encoded by transcripts lacking or including all alternatively spliced sequences (CD45R0) and longest (CD45RABC) isoforms are encoded by transcripts lacking or including all alternatively spliced sequences.

Figure 1 Crystal structure of the human CD45 ECD d1–d4 region. (a) Schematic representation of the full-length human CD45 sequence (top) and constructs used for functional studies and structural analyses (bottom). Colors indicate domains observed in the crystal structures (blue, d1; green, d2; yellow, d3; purple, d4). Asterisks mark residue numbering corresponding to the rat CD45 ortholog (rCD45d3d4). SP, secretion signal peptide; TM, transmembrane domain; P1 and P2, tyrosine-protein phosphatase catalytic domains. A, B and C indicate exons spliced out in the R0 isoform. (b) MALS analysis of CD45R0 (red), CD45RABC (blue) and CD45d1–d4 (purple). All constructs analyzed were monomeric in solution. Measured molecular weights versus theoretical masses (dotted lines) including the contribution of glycans are: 125.0 ± 0.5 versus 92.5 kDa for CD45R0; 79.0 ± 0.4 versus 68.0 kDa for CD45RABC; 64.6 ± 0.1 versus 62.7 kDa for CD45d1–d4, a.u., arbitrary units. (c) CD45d1–d4 crystal structure with individual domains color coded as in a. N-linked N-acetylgalactosamine (GlcNAc) moieties observed in electron density maps are shown in space-filling format. (d) Position of disulfide bonds (roman numerals) in the structure; yellow spheres correspond to sulfur atoms. (e) Cartoon representations of individual CD45 ECD domains with β-strands rainbow-colored to indicate β-strand sequence: N terminus, blue; C terminus, red. The β3 and β4 strands are missing in d1 and truncated in d2 (dotted ellipses). (f) Model of N-glycosylated CD45d1–d4, assuming glycosylation at sites marked with asterisks. Glycans at the sites marked with asterisks.

and have mucin-like regions of 41 and 202 residues, respectively (Fig. 1a). We expressed, in mammalian cells, the intact ECDs of human CD45R0 and CD45RABC along with subregions comprising d1 and d2 (CD45d1d2), d1–d3 (CD45d1–d3) and d1–d4 (CD45d1–d4), and rat CD45 d3 and d4 (rCD45d3d4) (Fig. 1a and Supplementary Fig. 1a). Multiangle light-scattering (MALS) analysis indicated that CD45d1–d4 and the CD45R0 and CD45RABC ECDs are monomeric in solution at concentrations of ~10 μM (Fig. 1b). We determined the crystal structures of CD45d1–d4 (to 2.9 Å resolution), CD45d1–d3 (3.1 Å resolution), CD45d1d2 (2.7 Å resolution) and rCD45d3d4 (2.8 Å resolution) (Supplementary Fig. 1b–d and Supplementary Table 1). CD45d1–d4 (Fig. 1c) comprises a ‘beads on a string’ array of four modular domains stabilized by eight disulfide bonds, seven forming part of the core of each domain and one at the d1–d2 interface (Fig. 1d). Inspection of the lattice did not reveal any potentially important oligomerization interfaces, consistent with the MALS data and with CD45 diffusing as monomers at the T cell surface.

### References

1. The CD45 ECD was predicted to comprise an N-terminal mucin-like region followed by a cysteine-rich domain (d1) and fibronectin type 3 (FN3) domains 2–4 (d2–d4).

2. Human CD45 isoforms arise from alternative splicing of exons 4, 5 and 6 of the PTPRC gene, which alters the length of the mucin-like region.

3. The smallest (CD45R0) and longest (CD45RABC) isoforms are encoded by transcripts lacking or including all alternatively spliced sequences.

4. In this study we show that the extracellular domain (ECD) of CD45 is rigid and extends beyond the distance spanned by TCR-ligand complexes, which explains why it will be excluded from sites of TCR-ligand engagement.

5. Live-cell imaging reveals the formation of submicron-scale structures that have several of the properties of close-contact zones postulated by the KS model.

6. The formation of these structures, which we call close contacts, is capable of initiating signaling even when TCR ligands are absent. Together these findings identify the structural basis for, and the unexpectedly potent signaling effects of, local CD45-kinase segregation.

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9. Measured molecular weights versus theoretical masses (dotted lines) including the contribution of glycans are: 125.0 ± 0.5 versus 92.5 kDa for CD45R0; 79.0 ± 0.4 versus 68.0 kDa for CD45RABC; 64.6 ± 0.1 versus 62.7 kDa for CD45d1–d4, a.u., arbitrary units.

10. CD45d1–d4 crystal structure with individual domains color coded as in a. N-linked N-acetylgalactosamine (GlcNAc) moieties observed in electron density maps are shown in space-filling format.

11. Position of disulfide bonds (roman numerals) in the structure; yellow spheres correspond to sulfur atoms.

12. Cartoon representations of individual CD45 ECD domains with β-strands rainbow-colored to indicate β-strand sequence: N terminus, blue; C terminus, red. The β3 and β4 strands are missing in d1 and truncated in d2 (dotted ellipses).

13. Model of N-glycosylated CD45d1–d4, assuming glycosylation at sites marked with asterisks. Glycans at the sites marked with asterisks.
Figure 2 The human CD45 d1–d4 region is rigid. (a) Comparison of the four crystal structures determined in this study. CD45d1–d4 is color coded as in Figure 1c. (b–d) Close-up views of the interdomain interfaces. Color coding is as in Figure 1c. Potential hydrogen bonds are shown as black dotted lines. Interface residues are labeled. (e) Surface representation of CD45d1–d4 showing the degree of conservation of surface residues for 11 mammalian CD45 family members. Residues are colored according to the number of identical or conservatively substituted residues at each position in a structural alignment: white, 0–6; pink, 7–8; red, 9–11 (Online Methods). CD45 sequences used for the alignment were: human (UniProtKB P08575); chimpanzee (UniProtKB H2Q09); gibbon (UniProtKB G1S8W0); rabbit, (UniProtKB G1T2D8); dog (UniProtKB F1Q1U1); ferret (UniProtKB M3YGB4); cat (UniProtKB M3WN30); panda (UniProtKB G1M263); cow (UniProtKB F1MJS9); mouse (UniProtKB P06800); and rat (UniProtKB P04157).

Figure 3 Axial dimensions and positioning of the CD45 ECD at a model surface. (a) Schematic representation of the CD45R0 ECD fused with Sema6A. Dashed lines indicate the mucin-like region. (b) Example class sums (from a total of 28) derived from the Sema6A-CD45R0 images obtained by negative-staining EM. Scale bar, 25 nm. (c) Two views of a 3D reconstruction fitted with the Sema6A domain and CD45d1–d4, with the mucin-like region modeled between them. (d) Left, principle of VA-TIRFM. Varying the angle of incidence (from $\theta_1$ to $\theta_2$) under total internal reflection conditions changes the depth of the evanescent field illuminating the sample. The resulting change in fluorescence signal has a magnitude that depends on the distance between the fluorescent object and the surface. The ECDs of CD45RABC, CD45R0 and CD45d1–d4 are shown (left to right); red and gray circles depict fluorescing and nonfluorescing dyes, respectively. Right, variation in fluorescence intensity at different incidence angles for N-terminal fluorescing labeled CD45RABC, CD45R0 and CD45d1–d4 attached to a SLB versus that of a fluorescent lipid in an adjacent bilayer. Data shown are the means for three repeats on different bilayers. (e) Dimensions of CD45R0 compared to those of a ternary CD4-TCR–MHC class II complex (PDB 3T0E) and a model of the CD2-CD58 complex (using CD2 (PDB 1HNF) superimposed on the CD2-CD58 d1-d1 complex (PDB 1QA9)) based on crystallographic data and shown in surface representation (only O-linked glycans are modeled on CD45R0).
The CD45 d1–d4 region is rigid

The two copies of CD45d1–d4 that make up the asymmetric unit were essentially identical (r.m.s. deviation of 1.5 Å for 346 Cα positions), suggesting that CD45d1–d4 is rigid. Comparisons with CD45d1d2, CD45d1–d3 and rCD45d3d4, from five crystal environments (Supplementary Table 1) supported this, as there were fewer than 5 degrees of rotational freedom between each domain pair (Fig. 2a). The domains are connected by very short and, for the d1–d2 and d3–d4 interfaces, proline-rich linkers, allowing extensive interdomain interactions (average surface area buried is 345 Å² versus 257 Å² for RPPMu24 (Fig. 2b–d). The d1–d2 interface is mainly hydrophobic, with phenylalanine and tyrosine side chains clustered at its center (Y237 and F246 from d1, and F329, Y371 and F376 from d2) (Fig. 2b). This arrangement is stabilized by a salt bridge formed by E273 and K303 at the interface edge and by a centrally positioned disulfide bond between C274 and C331 (bond III in Fig. 1d). In contrast, a centrally located arginine (R467 from d3) forming a network of hydrogen bonds and a salt bridge with d2 residues stabilizes the d2–d3 interface (Fig. 2c). The d3–d4 interface is dominated by a hydrophobic stacking interaction between Y450 of d3 and P512 of d4 supported by hydrogen bonding between the main chain oxygen of N510 and main chain nitrogen of A481 (Fig. 2d).

Sequence conservation in the mammalian CD45 ECD is extremely low (<15% identity), contrasting with the high identity (>90%) in, for example, type II RPTP ECDs25. No conserved surfaces in domains 1–4 are likely to bind ligands, and the limited conservation we observed (Fig. 2e) maps mostly to the three domain interfaces, which suggests that ECD rigidity is characteristic of CD45. However, conserved networks of residues also cap the ends of CD45d1–d4 (Fig. 2e and Supplementary Fig. 2d,e). Hydrophobic interactions involving I233, L250, V252, V256 and I281, along with a conserved disulfide (C226-C286) and hydrogen bonding of K229 and Y230 with E254 might help position the mucin-like extension at the N terminus of the d1–d4 region (Supplementary Fig. 2d). Similarly, a hydrophobic core comprising V491, M499, V501, L518, V520, F537, L542 and F550 and hydrogen bonding of S495-S498, Q543-Y548 and T546-T570 rigidify the base of the molecule, stabilizing D496 and a conserved negatively charged surface, which may influence ECD orientation at the cell surface (Supplementary Fig. 2e). A final notable feature is that whereas FN3 domains often lack cysteines, domains 1–3 of CD45 in a variety of species contain an average of 3–5 cysteines per domain (range 0–7), which are generally conserved among mammalian orthologs but not across phyla (data not shown). Assuming they form disulfides, the accumulated cysteines would also rigidify the CD45 ECD.

Axial dimensions of the CD45 ECD

We extended our structural analysis using single-particle negative staining electron microscopy (EM) (Supplementary Fig. 3a). To aid visualization, we fused the intact CD45R0 ECD with the globular ~70-kD ECD of the surface receptor Sema6A (PDB 3OKW) (Fig. 3a). Class averages for the resulting Sema6A-CD45R0 chimera (Fig. 3b and Supplementary Fig. 3b) resolved the FN3 domain array and Sema6A, with linking density corresponding to the mucin-like region. A three-dimensional (3D) reconstruction of the data to 33 Å resolution could be fit directly with the CD45d1–d4 and Sema6A crystal structures, but the mucin region was not resolvable, owing to limited map resolution (Fig. 3c and Supplementary Fig. 3c–e). Because Sema6A was positioned consistently enough for reconstruction, however, these data indicate that the d1–d4 mucin-like region is rigid, as expected for mucin-like structures of this length25. At ~80 Å long, the mucin-like region is more compact than for typical mucins (~2.5 Å per residue)25 but similar to CD43 (~2 Å per residue).26 At ~216 Å from N to C terminus, the CD45R0 ECD is smaller than suggested by rotary-shading experiments27 but matches predictions based on domain assignments.26 Sema6A-CD45RABC did not bind carbon-coated grids and was not analyzed by EM.
To investigate whether CD45 segregation from sites of TCR-ligand engagement might be caused by its ‘upright’ position at the cell surface, we tested whether the CD45 ECD lies prone or projects away from supported lipid bilayers (SLBs) using an angle-dependent total internal reflection fluorescence microscopy (VA-TIRFM) (Fig. 3d), which discriminates between the mean axial positions of fluorophores above a surface. We placed fluorescent tags at the N termini of CD45 ECDs and recorded the integrated fluorescence from the ECDs anchored to SLBs as a function of incident angle, normalized against that of a labeled lipid in adjacent SLBs (Fig. 3d). The different slopes obtained after plotting fluorescence versus incident angle could be attributed to differences in the axil height of the proteins, with the mean label positions of the CD45RABC and CD45SR isoforms experiencing substantially different evanescent fields versus CD45d1–d4, whose dimensions are similar to those of TCR-peptide-MHC class II-CD4 and CD2-LFA-3 complexes (Fig. 3e). The scale of the differences in slope (>1.6-fold) correlated with the structural data, which supports the hypothesis that to maximize the entropy of the ECD-SLB system wherein the ECDs are flexibly linked to the SLB, the ECDs are on average upright, with CD45RABC and CD45SR0 extending well beyond the distance spanned by TCR-ligand complexes.

**Signaling at close contacts driven by CD45 segregation**

The structural data implied that the mechanical properties of the CD45 ECD are important for its function, consistent with the idea that the ECD drives CD45 from sites of TCR-ligand engagement. However, CD45-kinase segregation has previously been observed only during synapse formation, too late to affect TCR triggering. We therefore characterized CD45 organization at very early stages of T cell contact formation and tested whether ECD-dependent CD45-Lck segregation coincides with and is required for TCR signaling.

We studied CD45 organization on live Jurkat T cells with TIRFM using fluorescent anti-CD45 Fab fragments to label the phosphatase. High cell activity (changes in surface and/or shape) at 37 °C necessitated data collection at 20 °C. Resting Jurkat T cells first placed on glass coverslips coated with nonactivating antibody (bovine IgG) formed multifocal contacts with the surface. Rather than being homogeneously distributed, CD45 was spontaneously excluded at each point of contact, forming ring-like arrangements (Fig. 4a). The contacts expanded to diameters >1 μm in ~200 s (Fig. 4a), relied on close membrane-surface apposition (Fig. 4b), showed strong actin labeling (Fig. 4c) and formed in an actin-dependent manner (Fig. 4d). J.RT3-T3.5 cells, which are derived from Jurkat cells and lack TCRs, also formed contacts (Fig. 4c), indicating that contact formation was TCR independent. CD45 was sterically excluded, as a lack TCRs, also formed contacts (Fig. 4c).
similar contacts (Fig. 4g), as did T cells interacting with glass surfaces coated with activating anti-CD3ε (OKT3) (Supplementary Fig. 4a). Given their similarity to the close-contact zones invoked by the KS model, we call these structures close contacts. Unexpectedly, whereas nonlymphoid cells (for example, human embryonic kidney (HEK 293T) cells) formed sinuous contacts with glass substrates (Fig. 4h), they formed robust T cell–like close contacts upon expression of a glycoalyx comprised of CD45RABC lacking a cytoplasmic domain (Fig. 4i).

To test whether CD45-Lck segregation observed at close contacts coincides with and is required for signaling, we engineered forms of mLck that would leave close contacts with CD45 (Fig. 5a,b) and tested their ability to reconstitute TCR signaling in Lck-deficient J.CaM1.6 cells contacting OKT3-coated surfaces. At the time of calcium signaling under these conditions (~2 min after contact; Supplementary Fig. 4a), a fluorescent membrane-spanning form of mLck (TMLck) was not excluded from close contacts formed with the OKT3-coated surface, producing anticorrelated fluorescence line profiles versus CD45 (Fig. 5c). However, attaching the CD45R0 ECD to TMLck (to form R0Lck), strongly altered the distribution of the kinase after surface contact (Fig. 5d). Overall, the contacts could be divided into two classes corresponding to patterns formed by ‘short-form’ Lck (mLck and TMLck) and ‘long-form’ Lck (RABCLck, R0Lck and d1–d4Lck) (Supplementary Fig. 4a,c–f and Supplementary Table 2). Whereas short-form Lck proteins and CD45 gave poorly correlated signals (Fig. 5e and Supplementary Fig. 5a) the spatial distributions of CD45 and long forms of Lck were well correlated (Fig. 5f, Supplementary Fig. 5b and Supplementary Table 2). ECD size and signaling were also strongly correlated (Fig. 5g and Supplementary Videos 1 and 2). OKT3 induced calcium responses in >50% of J.CaM1.6 cells expressing TMLck upon contact, whereas significantly fewer cells expressing RABCLck, R0Lck and d1–d4Lck responded (<30%; $P < 0.001$), and these responses were not significantly different from those of J.CaM1.6 cells. The dependence of signaling on Lck dimensions, observed also at 37°C (Supplementary Fig. 4g) revealed that receptor triggering requires the CD45 ECD–dependent segregation of the phosphatase and Lck at close contacts.

**Ligand-independent TCR triggering at close contacts**

The finding that CD45 and Lck spontaneously segregate on bovine IgG–coated glass surfaces offered a test of whether CD45-kinase segregation is sufficient to effect TCR triggering, which discriminates between the KS model and strictly ligand-dependent mechanisms of receptor triggering. At 37°C, IgG–coated surfaces induced calcium signaling by primary CD4+ and Jurkat T cells (49% and 68% of cells activated, respectively) (Supplementary Fig. 4h). These responses were comparable to those induced by OKT3 (83% and 90%, respectively) (Supplementary Fig. 4h–j). J.RT3-T3.5 cells, which lack TCR, were less responsive, indicating that local CD45-Lck segregation initiates TCR-dependent, ligand-independent signaling. Using SLBs to approximate native cell–cell interactions, we next investigated whether
CD45-Lck segregation resulted in TCR triggering per se, i.e., receptor phosphorylation. We initiated T cell–SLB contact by insertion of the small adhesion protein rat CD2 into SLBs at physiological densities (100–300 molecules per µm²) and by using Jurkat T cells expressing a nonsignaling form of CD48 (T92A) that binds rat CD2 with an affinity similar to that of human CD2-CD58 interactions (~20 µM)²⁹. Contacts formed rapidly and were characterized by the accumulation of fluorescently labeled CD42 under the cell and, invariably, CD45 exclusion (Fig. 6a, Supplementary Fig. 6a and Supplementary Video 3). Typical contacts (Fig. 6a, top) reached 1–3 µm in diameter in 10 s and expanded to 8–12 µm within 3–5 min. Occasionally, multifocal contacts also formed (Fig. 6a, bottom). CD45 exclusion was dependent on CD2-CD48 binding (Supplementary Video 4) and independent of the fluorescent labels used (Supplementary Fig. 6b). However, we did not observe exclusion from contacts of CD45 lacking its ECD (HA-CD45) (Fig. 6b and Supplementary Fig. 6c), the TCR (Fig. 6c and Supplementary Fig. 6d) or mLck (Fig. 6d, Supplementary Fig. 6f and Supplementary Video 3). The extent of CD45/Lck segregation was quantified ~10 min after contact formation (Fig. 6e). Assuming copy numbers of ~200,000 for CD45 (ref. 3) and 40,000 for mLck, we found that the CD45/Lck ratio decreased from 5:1 in resting cells to 2:3:1 at the T cell–SLB contacts.

The change in CD45/mLck ratio on T cell–SLB contact was accompanied by calcium signaling (Fig. 6f) at levels approaching those induced by immobilized OKT3 (typically 80–90% of cells responding) (Supplementary Fig. 4g,h). The response was significantly reduced by HA-CD45, as observed for direct TCR stimulation (Fig. 6f). Jurkat T cells expressing CD48 but lacking TCRs, Lck or T cell signaling protein Zap70 were also less or not responsive (Supplementary Video 5). Within 2 min of interaction between CD48-expressing Jurkat T cells and SLBs containing slightly higher levels of CD2 (~1,000 molecules/µm²), small clusters of fluorescently labeled Zap70 molecules accumulated at the cell-SLB contact (Fig. 7a and Supplementary Video 5). Significantly fewer clusters accumulated in TCR-deficient cells (Fig. 7b,c and Supplementary Video 6) although they appeared at similar times (data not shown). The fluorescence intensities (i.e., the sizes) of the Zap70 clusters were greater in Jurkats than in TCR-deficient cells (Fig. 7d) and increased during the first 180 s of contact (Fig. 7e). Because Zap70 recruitment depends strictly on TCR phosphorylation, these findings indicate that the TCR is triggered on the SLBs in a contact- and Lck-dependent but ligand-independent manner. Finally, in contrast to observations in SLBs containing TCR ligands, Zap70 cluster movement was not centripetal (Supplementary Fig. 7), which suggests that immunological synapse formation is not mobilized under these conditions. The sequence of events proposed to result in ligand-independent TCR triggering is illustrated in Supplementary Video 7.

**DISCUSSION**

To better understand how CD45 might influence receptor triggering, we determined the structure of its extracellular domain. The relationship between the structure and function of the ECD of CD45 is illuminated by comparisons with those of RPTPζ and RPTPσ, two large type II RPTPs²⁴,²⁵. The organization and dimensions of the rigidly arranged FN3 domains of CD45 are comparable to those in RPTPζ but the domain interfaces are larger and the d1 and d2 topologies are unique. In highly flexible RPTPs the domain interfaces are generally small or nonexistent. The degree of CD45 sequence conservation is very low compared to RPTPζ. The only conservation beyond that required for FN3 domain integrity is at domain interfaces influencing ECD shape and rigidity, at the top of d1 of the ECD, perhaps affecting positioning of the mucin-like region, and at the base of d4, possibly influencing interactions with the membrane. The CD45 ECD is also richer in cysteines likely to rigidify the protein by forming disulfide bonds than any other FN3-like domain-containing protein, including RPTPζ and RPTPσ. Overall, it seems that the mechanical properties of the ECD are most important to the functioning of CD45. The apparently rigid mucin-like region of CD45R0 extends the molecule to 216 Å. Assuming that the dimensions of TCR-ligand complexes are comparable to those determined crystallographically (~150 Å)²³,²⁴, even the smallest CD45 isoform will be sterically excluded from sites of TCR-ligand engagement if CD45 stands upright, as our VA-TIRF experiments suggest. Otherwise, the presence of CD45 would deform the lipid membranes in the contact region, increasing the energy of the system, as discussed elsewhere²⁵.

We also show that at regions of contact with artificial and model cell surfaces, T cells form close contacts characterized by ECD-dependent CD45 exclusion. To routinely observe multifocal close contacts on glass substrates it was necessary to image at 20 °C. These structures may have gone undetected previously because they are generally short-lived at 37 °C and more difficult to observe with the higher activity cells show at this temperature. At the time of signaling initiated by antibodies on these surfaces, the close contacts had diameters...
<1 μm. In bilayers at 37 °C, mostly single large close contacts formed quickly in the presence of CD2 and CD48, which suggests that CD45 is passively excluded from any site where CD2-ligand sized complexes assemble, including TCR-ligand complexes. Close contacts formed on bilayers are therefore qualitatively similar to close-contact zones invoked by the KS model. Close contacts observed on glass surfaces are distinct from CD45-excluding TCR microclusters because they form independently of TCRs, vary in size and do not move centripetally. The larger contacts formed on bilayers differ from faces are distinct from CD45-excluding TCR microclusters because they form within a minute rather than 15–30 min and do not centrally accumulate TCRs. The processes driving initial close-contact formation probably correspond to F actin–rich pseudopods. Notably, nonlymphoid cells formed T cell-like close contacts upon expressing a single glycolycylx component, i.e., cytoplasmic domain–lacking CD45, suggesting that the appearance of a CD45-containing glyocalyx on primitive lymphoid cells during their evolution could have allowed their utilization of contact-sensitive, molecular segregation–based receptor signaling.

Spontaneous CD45-kinase segregation at close contacts coincided with strong ligand-independent, bona fide TCR triggering, revealing the extent to which CD45 and kinase segregation per se can effect receptor signaling. The Lck/CD45 ratio measured in close contacts formed on SLBs (~50%) was similar to that giving half-maximal phosphorylation of CD3 by Lck and CD45 attached to lipid bilayers (68%)22. This suggests that the ratio of Lck/CD45 activities in resting cells might be set at a level that ensures TCR phosphorylation is sensitive to local CD45 segregation. Global alterations in this ratio, achieved pharmacologically or with genetic interventions initiate spontaneous ligand-independent signaling in T cells6,7, and our work identifies a physiological counterpart for these effects. Because it can be triggered in the absence of ligands, the TCR cannot be strictly dependent on ligand–induced receptor oligomerization and/or conformational changes. It is also unlikely to depend on physical forces acting through it because, in our bilayer experiments, the receptor was triggered by CD45 and kinase segregation even while suspended between the T cell surface and the SLB by CD2 and CD48. The ability of the TCR to be triggered in this way blurs the distinction between receptors and adaptors or signaling hubs, such as LAT, that lack ECDs but are heavily phosphorylated during T cell activation. Another example is CD6, which functions as both receptor and signaling hub.

What safeguards T cells from signaling at close contacts when TCR ligands are absent? Close contacts formed in vivo are probably smaller and shorter-lived than those formed in the absence of the glyocalyx of antigen-presenting cells in our experiments, perhaps allowing at most only subthreshold signaling in the absence of TCR ligands. Moreover, coreceptor engagement is impossible without TCR ligands, also constraining signaling. However, TCR triggering in close contacts lacking ligands might nevertheless be important in certain circumstances, for example, during β-selection, when the TCR has to signal from the cell surface in a partially assembled form (the ‘pre-TCR’), and does so even after its extracellular domains have been removed40. Conceivably, the threshold for TCR activation is fine-tuned by variation in the CD45 ECD during T cell development and differentiation.

In conclusion, we have shown that CD45 spontaneously segregates from Lck when T cells interact with lipid bilayers and the intersurface distance is set by complexes of adhesion proteins (CD2 and CD48) whose dimensions are comparable to those of TCR-ligand complexes. We showed that the exclusion of CD45 at close contacts is explained by differences in the dimensions of CD45 and these complexes and demonstrated that this is sufficient to trigger the TCR and initiate signaling even when TCR ligands are absent. As has been proposed8, similar but smaller or more dynamic close contacts formed in vivo that would otherwise sustain tonic or subthreshold signaling at best, TCR ligands might increase the probability of signaling simply by holding the receptor in the close contacts where encounters with Lck are favored over those with CD45.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Protein Data Bank: Structural data have been deposited under accession codes 5FMV (CD45d1–d4), 5FN6 (CD45d1–d3) 5FN7 (CD45d1d2) and 5FN8 (rCD45d3d4).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

V.T.C., R.A.F., K.A.G., S.F.L., E.Y.J., R.J.C.G., D.K., A.R.A. and S.J.D. designed experiments; V.T.C., R.A.F., K.A.G., S.F.L., J.M., P.J., M.P., K.H., C.S., H.C., Y.L., E.H., R.J.C.G. and A.R.A. performed experiments; R.A.F., K.A.G., S.F.L., C.S., E.Y.J., R.J.C.G., V.T.C., A.R.A., D.K. and S.J.D. drafted and/or edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

CD45 ECD constructs for crystallization and electron microscopy. For crystallization experiments fragments of the ECD of human CD45 (CD45d1–d4, residues 223–571; CD45d1–d3, residues 223–479; and CD45d1d2, residues 223–392 (UniProtKB P08075)) were amplified from CD45RABC cDNA (a gift of A. Weiss) by PCR. The rCD45d3d4 fragment (residues 357–546, UniProtKB P04157) was amplified from rat thymus cDNA. For the human and rat CD45 constructs, His6 tags were encoded at their 3′ and 5′ ends, respectively, to aid purification. The CD45d1d2 and CD45d1–d3 constructs were ligated into the pHLSec vector41 downstream of sequence encoding the chicken RPTPσ signal peptide (cRPTPσSP). The CD45d1–d4 and rCD45d3d4 constructs were inserted into the pE1E4 vector42 downstream of sequence encoding cRPTPσSP. For EM analysis the ECD of CD45R0 (residues 24–31 and 193–575 of UniProtKB P08075) was amplified from codon-optimized synthetic CD45RABC cDNA (Eurolens Genomics) and fused at the 5′ end to the ECD of mouse Sema6A (residues 19–571, UniProtKB O35464) amplified from Sema6A cDNA (a gift of R.A. Robinson) using PCR. The construct encoding Sema6A-CD45R0 was then subcloned into the pHF vector43 downstream of sequence encoding cRPTPσSP.

CD45d1d2 and CD45d1–d3 were expressed transiently in 293T cells, CD45d1–d4 and rCD45d3d4 were stably expressed in Chinese hamster ovary cells, and Sema6A-CD45R0 was expressed lentivirally in 293T cells. Kifunensine added to the cultures facilitated protein deglycosylation as required44. Proteins were purified using metal-chelate and size-exclusion chromatography. For crystallization the proteins were also de-tagged, de-glycosylated with endoglycosidase F1 and re-urctiated by lectin-affinity chromatography45. CD45d1–d4 was twice methylated46 for crystallization. See Supplementary Figure 1a for SDS-PAGE analysis of the soluble proteins.

Constructs for TIRFM imaging and signaling studies. Codon-optimized cDNA encoding ECD fragments of CD45 (CD45RABC, residues 24–575; CD45R0, residues 24–31 and 193–575; and CD45d1–d3, residues 223–571) was ligated into pHLSec downstream of sequence encoding cRPTPσSP and an HA tag. DNA encoding the murine CD4 transmembrane region (residues 1–509, UniProtKB P06240) by PCR. The first residue of the kinase was the pHLsec vector41 downstream of sequence encoding the chicken RPTPσ. Constructs for TIRFM imaging and signaling studies.

Supplementary Figure 1a

Constructs for measuring CD45 length using VA-TIRFM. HaloTag sequence encoding HaloTag. Cell lines expressing the lentiviral Construct for measuring CD45 length using VA-TIRFM. HaloTag sequence encoding the chicken RPTPσ. For EM analysis the ECD of CD45R0 (residues 24–31 and 193–575 of UniProtKB P08075) was amplified from codon-optimized synthetic CD45RABC cDNA (Eurolens Genomics) and fused at the 5′ end to the ECD of mouse Sema6A (residues 19–571, UniProtKB O35464) amplified from Sema6A cDNA (a gift of R.A. Robinson) using PCR. The construct encoding Sema6A-CD45R0 was then subcloned into the pHF vector43 downstream of sequence encoding cRPTPσSP. For EM analysis the ECD of CD45R0 (residues 24–31 and 193–575 of UniProtKB P08075) was amplified from codon-optimized synthetic CD45RABC cDNA (Eurolens Genomics) and fused at the 5′ end to the ECD of mouse Sema6A (residues 19–571, UniProtKB O35464) amplified from Sema6A cDNA (a gift of R.A. Robinson) using PCR. The construct encoding Sema6A-CD45R0 was then subcloned into the pHF vector43 downstream of sequence encoding cRPTPσSP. For EM analysis the ECD of CD45R0 (residues 24–31 and 193–575 of UniProtKB P08075) was amplified from codon-optimized synthetic CD45RABC cDNA (Eurolens Genomics) and fused at the 5′ end to the ECD of mouse Sema6A (residues 19–571, UniProtKB O35464) amplified from Sema6A cDNA (a gift of R.A. Robinson) using PCR. The construct encoding Sema6A-CD45R0 was then subcloned into the pHF vector43 downstream of sequence encoding cRPTPσSP.

CD45d1d2 and CD45d1–d3 were expressed transiently in 293T cells, CD45d1–d4 and rCD45d3d4 were stably expressed in Chinese hamster ovary cells, and Sema6A-CD45R0 was expressed lentivirally in 293T cells. Kifunensine added to the cultures facilitated protein deglycosylation as required44. Proteins were purified using metal-chelate and size-exclusion chromatography. For crystallization the proteins were also de-tagged, de-glycosylated with endoglycosidase F1 and re-urctiated by lectin-affinity chromatography45. CD45d1–d4 was twice methylated46 for crystallization. See Supplementary Figure 1a for SDS-PAGE analysis of the soluble proteins.

Crystall structure determination, refinement and analysis. The CD45d1d2 and rCD45d3d4 structures were determined by MAD analysis. Heavy atom positions were determined using SHELEX47, and solutions were input into autoSHARP48 for phase calculation, improvement and extension using higher-resolution native data to 2.3 Å and 2.45 Å, respectively. Map quality was excellent (Supplementary Fig. 1b–d), allowing tracing of the complete polypeptide chains, with electron density also observable for most residual N-linked glycans (Supplementary Fig. 1b–d). Initial models were automatically built using ARP/wARP49, followed by iterative rounds of manual model building in Coot50 and refinement in BUSTER51, REFMAC52 and Phenix53. CD45d1–d3 was solved by molecular replacement using CD45d1d2 as a search model in Phaser54. The model was completed by iterative rounds of manual building in Coot and refinement in BUSTER, applying noncrystallographic symmetry restraints. Supplementary Table 1 shows refinement statistics and protein geometry analysis (using Molprobity55). Sequence and structural alignments were performed in ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2) and SHP56, respectively; for Figure 2e, conservative substitutions were defined using the following residue groupings: IV/VM/FY/NDEBZ. Structure comparisons were made using DALLI (http://ekhidna.biocenter.helsinki.fi/dali_server). Protein interfaces were analyzed using PDBePISA (http://www.ebi.ac.uk/pdbe/prot_int/pistart.html). Structural figures were prepared using the PyMOL Molecular Graphics System, version 1.6 (Schrödinger, LLC).

Electron microscopy. Three microliters of 10 μg/ml Sema6A-CD45R0 was placed on a carbon-coated 300 mesh copper grid (Agar Scientific) for uranyl formate staining57. Images were collected using an FEI T12 electron microscope operating at 80 kV and nominal magnification 67,000×. Data were captured on a charge-coupled device (CCD) camera, and individual particle images interactively selected using Boxer (EMAN software63; Supplementary Fig. 3a shows representative data). In total 9,079 particles were selected and subjected to image analysis in IMAGIC58. After iterative centering to the sum of all images, a single round of multivariate statistics analysis and classification yielded 50 class averages. Supplementary Figure 3b shows all 50 class averages and corresponding ‘1-images’. The 1-images are a statistical measure of the consistency of features within class sums. Whereas in ‘S-images’ high densities correspond to the most significant image areas and are calculated as

$$S\text{-image} = \text{(squares of the average)}/\text{(average of the squares)}$$

an 1-image gives the amount of information collected in the different parts of the images in bits:

$$I\text{-image} = 2\log((n-1)\times S\text{-image})/(1-S\text{-image})$$

Twenty-eight class averages were used for 3D reconstruction of Sema6A-CD45R0, also in IMAGIC, based on angular reconstruction without a reference model. The resulting 3D map was filtered to 40 Å resolution and used in iterative projection matching-based reconstruction from the raw data images in
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NTIRF signals from the proteins were compared to that from SLBs containing (ammonium salt; DGS-NTA(Ni)), 95 wt% 1-palmitoyl-2-oleoylphosphatidylincidence, piezoelectric adjusters (Thorlabs), such that the angle of incidence, and therefore field penetration depth of the output from the 561-nm laser (4 W cm⁻²). Variation in the ~77°-79° range gave the greatest dynamic range for axial discrimination of the CD45 constructs. Field penetration depth was varied with a sawtooth-shaped waveform applied to the piezo amplifier (±150 V at 0.25 Hz).

Purified, histidine- and N-terminally HaloTag-tagged CD45 constructs, i.e., CD45d1–d4 and Sema6A and CD45d1–d4 including a modeled, glycosylated linker (Fig. 3c) and density calculated for this Sema6A-CD45R0 atomic model: at 33 Å the sema domain is absent (Supplementary Fig. 3e) and CD45 constructs. Field penetration depth was varied with a sawtooth-shaped waveform applied to the piezo amplifier (±150 V at 0.25 Hz).

VA-TIRF microscopy. Imaging was performed using a home-built TIRF microscope (described below) modified with a kinematic mirror mount with piezoelectric adjusters (Thorlabs), such that the angle of incidence, and therefore field penetration depth of the output from the 561-nm laser (4 W cm⁻²) could be varied (above the critical angle) in objective-type TIRF. Variation in the ~77°-79° range gave the greatest dynamic range for axial discrimination of the CD45 constructs. Field penetration depth was varied with a sawtooth-shaped waveform applied to the piezo amplifier (±150 V at 0.25 Hz).

Purified, histidine- and N-terminally HaloTag-tagged CD45 constructs, i.e., CD45d1–d4, CD45R0 and CD45RABC (0.5 µg/mL in 10 mM Tris, 150 mM NaCl, pH 8.0) labeled with HaloLigand TMR (Promega; GK251) were attached to nickelated lipids in SLBs formed as described below (5 wt% 1,2-dioleyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt; DGS-NTA(Ni)), 95 wt% 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) by incubation for 30 min before rinsing with buffer. VA-TIRFM signals from the proteins were compared to that from SLBs containing 0.1 wt% TRITC DHPE (N-(6-tetramethylrhodaminethiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine-amino, triethylammonium salt; Invitrogen) and 99.9 wt% POPC. Spatial variation in the TIRF excitation field intensity required normalization of sample signals against reference images corresponding to TRITC-labeled lipids in adjacent SLBs.

TIRF intensity, I, depends on distance to the fluorophore, z, and angle of incidence, θ, according to:

\[ I(z, \theta) = I_0(\theta) \exp(-z/d(\theta)) \]  

where \( d \) is the penetration depth (see ref. 28). Assuming collected light is proportional to \( I \), the detected intensity from fluorophores positioned at heights \( h_1 \) and \( h_2 \) above the surface is given by:

\[ I_r = \frac{\alpha I(\theta) h_2}{I(\theta) h_2 + \alpha} \exp(-h_2/h_1)(\theta) \]  

where \( \alpha \) is a factor correcting for differences in the surface fluorophore concentration in the paired samples. Measuring the change in intensity as a function of the angle of incidence allows \( \alpha \) to be eliminated, giving:

\[ \frac{dI_r}{d\theta}/I_r = \beta(h_1 - h_2), \text{where } \beta = \frac{1}{d^2(\theta)} \frac{d(d(\theta))}{d(\theta)} \]  

The average position of the fluorophore is measured in these experiments. For each protein the signal \( (d_1/d_2)/I_r \) varied versus the labeled SLB (0.21 per degree for CD45d1–d4, 0.33 per degree for CD45R0 and 0.35 per degree for CD45RABC). From equation (3) the average CD45R0 and CD45RABC signals were 157% and 166% higher than for CD45d1–d4, respectively, effects we attribute to differences in the axial heights of the proteins.

SLB preparation. SLBs were prepared as described with minor modifications. Glass coverslips (VWR International) were cleaned for 1 h in 3:1 sulfuric acid/hydrogen peroxide, followed by thorough rinsing and plasma cleaning for 15 min. SLBs were formed on the coverslips using press-to-seal silicone isolators (Grace Bio-Labs) by adsorption and subsequent rupture of lipid vesicles. The vesicles consisted of POPC with either 0 or 5.0 wt% 18:1 DGS-NTA(Ni) (both Avanti Polar Lipids, Ala haber USA) or 0.01 wt% Oregon Green 488, 1,2-dihexadeca-noyl-sn-glycero-3-phosphoethanolamine (OG-DHPE) in 10 mM HEPES, 150 mM NaCl, pH 7.4. After SLB formation the vesicle solution was replaced with buffer. For experiments described in Figures 6 and 7, 0.25 µg/ml rat CD2 with a H2SRAWHQFGHGHa ‘spacer-his’ tag was bound to the SLBs.

Cell culture. Plasmid transfection and lentivirus infections were used to generate cell lines expressing the constructs described above. For imaging, T cells were grown in phenol red-free RPMI supplemented with 10% FCS, 1% HEPES buffer, 1% sodium pyruvate and 1% penicillin-streptomycin (T cell medium).

TIRF imaging. Before imaging, T cells expressing HaloTag-tagged proteins, i.e., mLck, TMLck, RABLck, ROLck, d1–d4Lck, TCR-β, HA-CD45 and Zap70 were labeled with HaloLigand TMR for 30 min. The cells were then subjected to three PBS washes following by a 30 min incubation in T cell medium. Approximately 10⁶ cells were then resuspended in PBS and incubated with anti-CD45 (Gap8.3) antibody Fab fragments tagged with Alexa Fluor 488 at 22 °C. After this incubation, the cells were washed three times with PBS (involving centrifugation at 600 × g, 2 min) and transferred to coverslips that had been cleaned with an Argon plasma (PDC-002; Harrick Plasma, Ithaca NY) and then coated with OX7 (anti-rat Thy1.1) antibody only or a 9:1 mixture of OX7 and OKT3 (anti-CD3) antibodies (total 10 µg/mL, experiments shown in Figs. 4 and 5). Alternatively, the cells were allowed to settle on SLBs prepared as described above (experiments shown in Figs. 6 and 7). Imaging was carried out within the first minutes after cell attachment at temperatures indicated in the figure legends.

Imaging was performed using a home-built TIRF microscope. Diode lasers operating at 488 nm (Spectra Physics (Figs. 4, 6 and 7); iBeam, 488_1002; Topica (Fig. 5)) or 561 nm (Spectra Physics (Figs. 4, 6 and 7); iView, 561/04-01-0100-300; Cobolt (Fig. 5)), or a HeNe laser operating at 633 nm (Melles Griot) were directed into a TIRF objective (60× Plan Apo TIRF, NA 1.45, Nikon Corporation (Figs. 4, 6 and 7); N2709400, APON60X0 TIRF; Olympus (Fig. 5) mounted on an Eclipse TE2000-U microscope (Nikon Corporation (Figs. 4, 6 and 7)) or Olympus IX-71 microscope (Fig. 5) parallel to the optical axis and offset giving total internal reflection.

Emitted fluorescence was collected by the same objective and separated from the returning TIR beam by a dichroic (D101-R4-5/488/561/635; Semrock), split into blue and red (FF562-Di03; Semrock) components using a DVS (Photometrics) loaded with appropriate filters (BLP01-561R and EML01-R488-25; Semrock). Images were recorded on an EM-CCD camera (Photometrics, Roper Scientific; ~70 °C (Figs. 4, 6 and 7); Evolve 512; ~80 °C (Fig. 5)) either by alternating between laser excitation sources (561 nm, ~100 W cm⁻² and 488 nm, 25 W cm⁻² as measured by epifluorescence) using mechanical shutters (Prior Scientific) and an exposure of 400 ms (Fig. 5) or by simultaneously recording two-color fluorescence signals from 488- and 561-nm or 488- and 633-nm excitation (Figs. 4, 6 and 7) with each color recorded on half of the EMCCD chip. Regularly spaced ion-beam etched holes in gold-on-glass allowed image registration with a mean alignment precision of ~120 nm. Data were acquired using single snap shots or time-lapse acquisition (Figs. 5, 6 and 7; exposure time 100 ms, time between frames 1–5 s) using MicroManager. Quantitative analysis of CD45-Lck chimera segregation. Images were recorded at 33 frames per second for 400 frames, and the imaging stack averaged (mean) to give the final images. No significant changes in contact morphology were observed during this period at 20 °C. Image processing techniques for automatically locating close contacts via CD45 fluorescence proved unreliable because the exclusion zones were insufficiently uniform. Close contacts were therefore defined by ‘eye’ as an ‘s-sided by manually tracing the CD45 (Gap 8.3 Fab) fluorescence outlining each contact in ImageJ (US National Institutes of Health; http://imagej.nih.gov.vn). Approximate radii were calculated for each region from its circumference, and area values extracted assuming a circular distribution using

\[ \text{Radius} = \sqrt{\frac{\text{Area}}{\pi}} \]

\[ \text{Area} = \pi \times \left( \frac{\text{Radius}^2}{4} \right) \]
To quantify CD45 and CD45-Lck chimera segregation at close contacts (Fig. 5e,f), we measured the correlation between the CD45 and Lck images of the regions and contacts selected. Pixel intensities in the two channels were plotted against each other and a coefficient of determination ($R^2$) reported to a linear fit. This value was then plotted against contact radius size (short-form constructs, Supplementary Fig. 5a; long-form constructs, Supplementary Fig. 5b). For the short-form constructs, if Lck enters the close contacts, the images will not be correlated and the linear correlation will report a small value, close to 0. If long-form CD45-Lck chimeras are excluded from the close contacts, the images will correlate, giving a coefficient of determination closer to 1. The analysis will overestimate the correlation for small contacts as revealed by the broad distribution of $R^2$ values for small close contacts (Fig. 5e,f). The smallest zone measurable, defined by the Abbe diffraction limit, was 174 nm agreeing with our empirically determined pixel resolution = 178 nm/pixel. The minimum observable zone of differential exclusion would have a three-pixel diameter as confirmed by the absence of measurable contacts below ~600 nm (Fig. 5e,f).

**Quantitative analysis of Zap70 recruitment on SLBs.** Assuming a 5:1 CD45/Lck ratio outside the contact for a typical Jurkat T cell, CD45 and Lck molecules in the contacts, i.e., the binary mask created for the 488 nm channel was subtracted from the CD45 channel was subtracted from the binary mask created from the CD45 channel. A custom ImageJ macro detected individual cells at suitable time points. Intensities were imported to MATLAB (http://site.physics.georgetown.edu/matlab) for further analysis. Briefly, after filtering the data with a Savitzky-Golay moving average and subtracting the average fluorescence intensity between frame 1 and the frame corresponding to the maximum fluorescence intensity, a change in fluorescence intensity above 0 gave the ‘cell landing time.’ Signaling cells were identified when (i) the maximum intensity was twofold higher than the intensity recorded at the landing time and (ii) ‘triggering time’ (time of the largest subsequent fluorescence increase) was greater than landing time. After correcting for moving cells, the landing time, triggering time and triggered proportion were reported with an error of only 8% as determined by eye for 150 randomly chosen cells.

**Quantitative analysis of CD45-mLck segregation on SLBs.** Custom MATLAB code was used to analyze the image data. First, the cell area was manually identified using fluorescence in the 561 channel (Lck fluorescence). Binary masks were then generated corresponding to the selected cells in both the 488 and 561 channels to separate signal from background (Fig. 6e). Pixels were assigned a value of 1 if their intensity values fulfilled the condition $I > (\text{mean}(I_{\text{region}}) - 2 \times \text{std}(I_{\text{region}}))$. The binary mask created for the 488 nm (CD45 fluorescence) channel was designated to be the region of the cell surface excluded from SLB contact, and the mean intensity of the pixels corresponding to this area calculated for both the CD45 and Lck channels ($I_{\text{inside}}$). To create a binary mask describing the area of the cell corresponding to the SLB contact, the binary mask created from the CD45 channel was subtracted from the one created from the Lck channel and applied to both channels ($I_{\text{outside}}$). The CD45/Lck ratio in the contact was calculated from the fraction of total CD45 and Lck molecules in the contacts, i.e.,

$$f(CD45_{\text{tot,ln}}) = \frac{I_{\text{inside}}}{I_{\text{outside}}} = 0.41$$

and $f(Lck_{\text{tot,ln}}) = 0.88$.

Assuming a 5:1 CD45/Lck ratio outside the contact for a typical Jurkat T cell, this means that the ratio inside changes to $5 \times 0.41 \times 0.88 = 1.73$.

**Quantitative analysis of Zap70 recruitment on SLBs.** Zap70 recruitment was measured using time-lapse fluorescence image acquisition at a rate of 1 frame per second (exposure time of 500 ms). Bespoke MATLAB code was used to analyze the data. First, cell area was manually selected on the basis of bright-field images of the cells. Corresponding images of HaloLigand-TMR labeled Zap70 fluorescence were band-pass filtered, signals of signaling molecules detected using intensity and signal-to-noise thresholds, and their positions in subsequent frames linked to form trajectories using published particle detection and tracking algorithms adapted for MATLAB and further modified. Zap70 intensities were extracted by averaging maximum pixel intensity over multiple frames before bleaching occurred (i.e., before particle intensity in frame n + 1 dropped below 60% of particle intensity in frame n). Intensity values were then divided by the average background fluorescence of the given cell to account for cell-to-cell variation in Zap70-Halo expression (in the presence of endogenous Zap70). For analyzing the evolution of mean particle intensity over time, the median intensity of all Zap70 trajectories observed in a time window of 20 s was calculated from $t = 20 s$ after contact formation and the error estimated by bootstrapping (i.e., calculating the s.d. of the median values of 1,000 data sets created by randomly drawing samples of the size of the original data set).

**Statistical methods.** Data plotting and statistical analysis were performed using Origin 8.1 (OriginLab). Differences between sets of non-normally distributed data were assessed using Mann-Whitney U-tests.