Polarized release of T-cell-receptor–enriched microvesicles at the immunological synapse

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The recognition events that mediate adaptive cellular immunity and regulate antibody responses depend on intercellular contacts between T cells and antigen-presenting cells (APCs)1. T-cell signalling is initiated at these contacts when surface-expressed T-cell receptors (TCRs) recognize peptide fragments (antigens) of pathogens bound to major histocompatibility complex molecules (pMHC) on APCs. This, along with engagement of adhesion receptors, leads to the formation of a specialized junction between T cells and APCs, known as the immunological synapse2, which mediates efficient delivery of effector molecules and intercellular signals across the synaptic cleft1.

T-cell recognition of pMHC and the adhesion ligand intercellular adhesion molecule-1 (ICAM-1) on supported planar bilayers recapitulates the domain organization of the immunological synapse3,4, which is characterized by central accumulation of TCRs5, adjacent to a secretory domain6, both surrounded by an adhesive ring4,5. Although accumulation of TCRs at the immunological synapse centre correlates with T-cell function4, this domain is itself largely devoid of TCR signalling activity4,5, and is characterized by an unexplained immobilization of TCR–pMHC complexes relative to the highly dynamic immunological synapse periphery6,5. Here we show that centrally accumulated TCRs are located on the surface of extracellular microvesicles that bud at the immunological synapse centre. Tumour susceptibility gene 101 (TSG101)6 sorts TCRs for inclusion in microvesicles, whereas vacuolar protein sorting 4 (VPS4)5,6 mediates scission of microvesicles from the T-cell plasma membrane. The human immunodeficiency virus polyprotein Gag co-opts this process for budding of virus-like particles. B cells bearing cognate pMHC receive TCRs from T cells and initiate intracellular signals in response to isolated synaptic microvesicles. We conclude that the immunological synapse orchestrates TCR sorting and release in extracellular microvesicles. These microvesicles deliver transcellular signals across antigen-dependent synapses by engaging cognate pMHC on APCs.

The nature of the biophysical environment that governs molecular domain organization at the immunological synapse remains unclear. Confinement of pMHC4, TCRs3,5 and cytoplasm (Supplementary Fig. 1) suggests that a general diffusion barrier separates TCRs and cytoplasm at the immunological synapse centre from the rest of the T cell. To better understand the basis for the observed central confinement of pMHC, TCRs and cytoplasm at the immunological synapse, we investigated CD4+ T-cell immunological synapse formation using high-resolution optical imaging by total internal reflection fluorescence microscopy (TIRFM), integrated with transmission electron microscopy (TEM) and electron tomography.

T cells from TcrAND transgenic mice4 form TCR microclusters in response to engagement by the cognate class II pMHC molecule I-Eκ complexed with the moth cytochrome C peptide MCC 88–103 (MCC–I-Eκ). Over approximately 10 min, TCR microclusters, together with bound pMHC4,6, are transported on the cell surface to the immunological synapse centre, where they are consolidated into an immobilized domain7. To follow ultrastructural changes associated with immunological synapse formation, TcrAND T cells were fixed after 5, 10, 15 and 20 min of interaction with supported lipid bilayers containing MCC–I-Eκ and ICAM-1, and imaged first by TIRFM and then by TEM. As a control, we used the non-cognate pMHC β2m–I-Eκ, which did not arrest motility or induce immunological synapse formation in TcrAND T cells (Supplementary Fig. 2a). TEM time series of TcrAND T cells forming immunological synapses on antigen-containing bilayers revealed changes in cell morphology that were characteristic of antigen-induced cell polarization (Supplementary Fig. 2b–d). Notably, at the 10 min time point, the centre of the T-cell contact interface showed an unexpected change in morphology, from a planar plasma membrane in continuous close apposition with the planar bilayer (Fig. 1a) to the appearance of numerous microvesicles (Fig. 1b and Supplementary Fig. 2e), approximately 70 nm in diameter (Supplementary Fig. 3a), that were contained within a central extracellular cavity (Fig. 1b). Microvesicle formation was antigen-specific, as they did not form with bilayers containing β2m–I-Eκ (Fig. 1a and Supplementary Fig. 2e), and could be modulated by the potency of the activating ligand, or by provision of costimulation10,11 (Fig. 1f and Supplementary Discussion). To visualize the distribution of microvesicles more clearly and verify their dissociation from the plasma membrane, we performed dual-axis tomography (Supplementary Video 1) on four serial sections through an immunological synapse, ranging from 150–250 nm in thickness. The associated three-dimensional model (Fig. 1c–e and Supplementary Videos 2 and 3) of the joined tomograms demonstrated that discrete extracellular microvesicles, with no connection to overlying plasma membrane (Supplementary Fig. 4a, b), predominate in the central cavity, along with occasional membrane projections and membrane buds of nascent microvesicles (Supplementary Fig. 4c–i).

Comparison of the distributions of TCRs and microvesicles at the immunological synapse demonstrated that they were spatially correlated (Supplementary Fig. 3b–d). To establish whether TCRs present at the immunological synapse centre were associated with microvesicles, we developed a novel method for optical–electron microscopy correlation, based on registration of TIRFM and corresponding TEM images of immunological synapses aligned using a microfabricated grid (Supplementary Fig. 5). Electron tomography of T–cell–bilayer interfaces confirmed the presence of microvesicles within a roughly circular extracellular cavity at the immunological synapse centre (Fig. 1h, i and Supplementary Videos 4 and 5). Optical–electron microscopy correlation

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then allowed us to assign molecular distributions to immunological synapse ultrastructure. This revealed that TCR fluorescence at the immunological synapse (Fig. 1g, j) corresponded almost exclusively to extracellular microvesicles contained within the cavity at the immunological synapse centre (Fig. 1l, n). The characteristic F-actin depletion at the immunological synapse centre (Fig. 1j, k) is also circumscribed by the plasma membrane boundary of the central cavity (Fig. 1o), reflecting interruption of cytoplasmic continuity at the immunological synapse centre. The annular ICAM-1–LFA-1 adhesive domain, which surrounds centrally accumulated TCRs (and associated MCC–I-E\textsuperscript{\beta}), is wholly contained within the plasma membrane border of the central cavity (Supplementary Fig. 6), which effectively precludes membrane-tethered LFA-1 from binding ICAM-1 at the immunological synapse centre. Our finding that TCRs are present in extracellular microvesicles also accounts of cytoplasmic continuity at the immunological synapse centre.
for the inability of centrally accumulated TCRs to exchange with TCRs elsewhere in the T cell (Supplementary Fig. 1a, b), and for the confined cytoplasmic diffusion observed at the immunological synapse centre (Supplementary Fig. 1c).

After 30–45 min, TcrAND T cells break immunological synapse symmetry and resume motility12. Migrating T cells left behind 80% of TCRs on the bilayer (Supplementary Figs. 7a, c and 8a and Supplementary Video 6). Optical–electron microscopy correlation of TcrAND T cells resuming motility after immunological synapse formation confirmed that these particles were extracellular TCR-enriched microvesicles released from the immunological synapse centre (Fig. 2a, b). The microvesicles diffused apart, which allowed us to characterize their composition by indirect immunofluorescence labelling and TIRFM (Supplementary Fig. 8b). TCR-enriched microvesicles were devoid of tyrosine phosphorylation (Fig. 2c), consistent with termination of TCR signalling. TCR-enriched microvesicles clustered MCC–I-Eκ, but not β2m–I-Eκ, on bilayers (Fig. 2d and Supplementary Figs. 7d and 9), establishing that TCRs were on the external surface of microvesicles and were capable of binding cognate pMHC.

To investigate the mechanism by which microvesicles were produced, we next studied the role of the endosomal sorting complex required for transport (ESCRT)–I member TSG101, which was previously implicated in ubiquitin-dependent sorting of TCRs to the immunological synapse centre. ESCRT–I proteins, such as TSG101, typically carry out vesicle budding and fission at limiting membrane-tethered microvesicles (Supplementary Fig. 8b). TCR-enriched microvesicles were devoid of TCRs, demonstrating that TSG101 was necessary for sorting of TCRs into microvesicles (Fig. 3a).

We next investigated the role of the ESCRT-III complex, which is responsible for the final steps in vesicle fission. We used primary human
CD4+ T cells which, like TcrAND murine T cells, produce TCR-enriched microvesicles at the immunological synapse centre (Supplementary Fig. 12). We transfected human CD4+ T cells with a construct encoding a dominant-negative mutant of human VPS4(E228Q), that prevents ATP binding, fused to green fluorescent protein (VPS4dn–GFP)13. Expression of VPS4dn–GFP disrupts the function of endogenous VPS413, which binds to and catalytically disassembles14 membrane-associated ESCRT-III oligomers and is thought to be obligatory for sustaining membrane budding reactions15,16. As a control, we transfected human CD4+ T cells with a construct encoding GFP alone. Control T cells formed microvesicles at the immunological synapse centre, the majority of which were separated from the limiting plasma membrane of the central cavity (Fig. 3b, d). In marked contrast, the immunological synapse of VPS4dn–GFP-transfected T cells contained nascent microvesicles, within a fragmented central compartment, which remained tethered to the limiting plasma membrane (Fig. 3b–d) at constricted bud necks (Fig. 3c). Consistent with the role of VPS4 'downstream' of early acting ESCRTs, VPS4dn–GFP did not affect TCR sorting to the immunological synapse centre (Supplementary Fig. 13), although its distribution at the immunological synapse was altered when compared to wild-type VPS4 (Supplementary Fig. 14). These observations indicate that VPS4 is involved in fission of TCR-rich membrane buds, thereby producing TCR-enriched microvesicles at the immunological synapse centre5,17.

The ESCRT pathway is also exploited by the human immunodeficiency virus (HIV) structural polyprotein Gag13,14 for membrane budding and release of virus particles from the plasma membrane of infected cells. This prompted us to test whether Gag might interfere with the biogenesis of TCR-enriched microvesicles in human CD4+ T cells. We expressed a construct encoding Gag fused to GFP (Gag–GFP) in primary human CD4+ T cells, and analysed immunological synapse formation by optical–electron microscopy correlation. Transfected T cells adhered poorly to supported bilayers containing ICAM-1 alone, with small Gag–GFP puncta present throughout the contact interface (Supplementary Fig. 15b, c). Notably, TCR engagement led to robust recruitment of Gag–GFP to the contact interface (Supplementary Fig. 15b, c), where it accumulated at the immunological synapse centre, displaced TCRs (Fig. 3f and Supplementary Fig. 15e, d), and resulted in the release of Gag–GFP-containing microvesicles, which are analogous to virus-like particles (VLP) that are produced during HIV infection (Fig. 3g, h and Supplementary Fig. 15f, g). Conversely, in T cells with low Gag–GFP expression, TCRs accumulated normally at the immunological synapse centre (Supplementary Fig. 15d, e), resulting in the release of TCR-enriched microvesicles (Fig. 3e and Supplementary Fig. 15f, g). The ESCRT-binding domain of Gag is required for its recruitment and central accumulation at the immunological synapse following TCR engagement (Supplementary Fig. 16 and Supplementary Discussion). Taken together, these findings indicate that Gag antagonizes ESCRT-dependent sorting of TCRs into microvesicles, and in this context TCR ligation directs the polarized budding of VLPs at the centre of the immunological synapse.

To verify that microvesicles have a physiological role in cellular interactions, we next asked whether TcrAND T cells produce TCR-enriched microvesicles in conjugates with antigen-bearing B cells. As expected, TcrAND T cells readily accumulated TCRs at the immunological synapse centre in conjugates with MCC-pulsed congenic B10.Br B cells (Supplementary Fig. 17a, b). In approximately 40% of T–B cell conjugates (Fig. 4a, b), small puncta of TCRs could be detected in B cells 'distal' to the immunological synapse, consistent with T cell to B cell synaptic transfer of TCR-containing microvesicles, whereas no T-cell polarization or TCR transfer was detected in conjugated B cells pulsed with...
β2m peptide (Supplementary Fig. 17a, b). Because TSG101 is critical for the production of TCR-enriched microvesicles in the planar bilayer model, we next investigated whether TCR transfer to live B cells is affected by siRNA-mediated suppression of TSG101. In conjugates of TSG101-suppressed TcRAnd T cells and MCC-pulsed B cells, transfer of TCRs was reduced by approximately 80% (Fig. 4a, b), relative to conjugates treated with control siRNA, despite effective TCR polarization to the immunological synapse (Supplementary Fig. 17b). As previously described, transfer of pMHC from B cells to T cells was also observed (Fig. 4a, c and Supplementary Fig. 17a–b). However, this pMHC transfer was unaffected by TSG101 silencing in the T cell (Fig. 4a, c), demonstrating that TSG101 selectively controls TCR transfer to B cells during bidirectional membrane exchange in T–B cell conjugates.

We next asked whether isolated microvesicles induce signalling in antigen-presenting B cells. Engagement of MHC II molecules on B cells triggers tyrosine phosphorylation and intracellular calcium signalling, by coupling to the B-cell antigen receptor signalling machinery, resulting in cell proliferation in primed B cells. We therefore asked whether B cells presenting cognate MCC–I-Ek complexes are activated by specific TCRs present on microvesicles. We introduced B cells, loaded with the Ca2+-sensitive dye Fluo-4, onto bilayers containing TCR-enriched microvesicles, from which T cells had been removed (Supplementary Fig. 17c), and monitored Ca2+ signalling by confocal microscopy (Fig. 4d and Supplementary Fig. 17d). B cells pulsed with MCC were motile on bilayers, but stopped and showed sustained increase in intracellular Ca2+ on encountering TCR-enriched microvesicle patches on bilayers (Fig. 4d, e). In contrast, B cells not loaded with MCC did not show an increase in Ca2+ relative to controls (that is, cells on bilayers with ICAM-1 alone, Fig. 4d, f). Thus, B-cell signalling by pMHC is initiated by cognate recognition of TCRs on microvesicles. In support of a role for TCR-enriched microvesicles during human T–B cell interaction, we found that Raji B cells, in superantigen-induced conjugates with human CD4+ T cells,22 received TCR puncta that activated phospholipase Cγ1 (PLCγ1), a key mediator of intracellular Ca2+ signalling (Supplementary Fig. 18).

Our finding that the immunological synapse centre is an extracellular cavity, filled with TCR-enriched microvesicles by an ESCRT-dependent mechanism, provides a fundamentally new model for supramolecular domain organization at the immunological synapse (Supplementary Fig. 19a). Shedding of TCRs in microvesicles constitutes a novel mechanism for TCR ‘downregulation’, following engagement by pMHC, that acts in parallel with receptor internalization.24 Our observations raise the possibility that other immune cells known to accumulate immunoreceptors at the synapse centre, such as B22 and NK26 cells, may also release them in microvesicles for intercellular communication. Cognate recognition of TCR-enriched microvesicles by pMHC on B cells may provide ‘help’ to B cells that is calibrated to the pMHC density present on their surface, pointing to a plausible mechanism for avidity-adjusted delivery of T-cell help to B cells.27 Finally, we find that TCR-enriched microvesicle biogenesis is a native pathway in T cells, triggered by antigen recognition, that may be co-opted by HIV proteins for polarized retroviral transmission at antigen-dependent immunological synapses (Supplementary Fig. 19b).

**METHODS SUMMARY**

Mature TcRAnd T cells were expanded with MCC peptide and IL-2, and used once quiescent. Human CD4+ T cells were isolated from peripheral blood by negative selection and used within 48 h. T cells clones were expanded for one cycle using heterologous irradiated peripheral blood mononuclear cells (PBMCs), phytohaemagglutinin (PHA) and IL-2 and used once quiescent. 1.2 dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) lipid bilayers containing 12.5 mol% 1,2-dioleoyl-sn-glycero-3-[(N-(6-amino-1-carboxyethyl)iminodiacetic acid)succinyl] (nicked salt) (DGS-NTA-NB) were deposited on coveirsheets that were cleaned with peroxidized sulphuric acid and rinsed with pure water. Bilayers contained 100 molecules per μm² MCC–I-Ek, and 200 molecules per μm² ICAM-1 unless otherwise stated. Optical imaging was performed using a Nikon TIRF microscope and an LSM510 confocal microscope. TEM imaging and tomography was performed using Phillips CM12.

**Online Content**

Any additional methods, Extended Data items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Methods

Proteins, peptides and antibodies. Soluble 1-εβ-2XHis, and ICAM-1-His3 were expressed in insect cells (S2) and isolated by affinity and fast protein liquid chromatography (FPLC) purification as previously described.6 All fluorophores for amine and maleimide coupling were purchased from Molecular Probes (Invitrogen). ICAM-1 was amine-labelled using SE-Cy5 or SE-AF405. MCC, K99A and β2m peptides were synthesized by fluorenylmethyloxycarbonyl (FMOC) chemistry and single AF488, AF633 and AF568 SE fluorophores were attached at their amino termini before side-chain deprotection, trifluoroacetic acid (TFA) cleavage from resin, and HPLC purification. Peptide labelling with fluorophores was confirmed by mass spectrometry and purity was >98%. Loading of peptides onto 1-εβ-2XHis, was performed under acidic conditions as previously described,7 and MCC-εβ-2XHis complexes purified by FPLC, yielding fluorophore to protein ratios of 0.7–1.1. HLA-DR4 was expressed purified, refolded in vitro with haemagglutinin peptide, and BirA biotinylated as previously described.8 Fab fragments of mouse TCR-β-specific antibody H57 were produced by papain cleavage and purified by ion-exchange chromatography.9 Fab’ fragments of human CD3ε-specific antibody UCHT1 were produced by papain cleavage to Fab’ fragments, reduced with 0.5 mM β-mercaptoethanol to yield Fab’ fragments with free (cysteine) thiol at their C termini, and conjugated to biotin-maleimide (Pierce) to yield monobiotinylated Fab. Mouse monoclonal antibody 2C11 was produced and used as the pericentor’s僖or, as per the vendor’s instructions for immunofluorescence labelling and western blots. Dynamin-related, linked in biotin, was used in conjunction with fluorescently labelled streptavidin (Invitrogen) to detect phosphatidylethanolamine, and fluorescently labelled annexin V (Invitrogen) was used to label phosphatidylserine. Secondary antibodies for infrared detection in western blots were from LI-COR Biosciences.

Mice, cells and cell purification. Mice used in this work were housed under pathogen-free conditions in the Skirball Institute Central Animal Facility in accordance with local and NIH regulations, and euthanized according to the guidelines of the Panel on Euthanasia of the American Veterinary Medical Association. TcrAND B10 and B10.Br mice were mated, and F1 progeny used for experiments at 6–8 weeks of age.9 Spleens and lymph nodes from F1 TcrAND mice were macerated and, following red blood cell lysis, incubated with 3 μM MCC peptide and 50 U recombinant human IL-2 in complete OK-DEMEM media to expand T cells. TcrAND CD4+ T cells were purified from cultures at day 4 of culture for siRNA suppression, or day 6 for all other experiments, by negative selection using mouse CD4+ T cell isolation kit II (Miltenyi Biotec) (purity was >95%). B cells were isolated from macerated spleens collected from B10.Br mice using anti-mouse CD43 and CD4 magnetic beads for negative selection (Miltenyi Biotec), yielding >98% CD19+ B cells. B cells were incubated overnight with 100 nM εβ-lipopolysaccharide (Sigma) and 50 μM of the indicated peptides for T-cell conjugation and Ca2+ experiments. Spleens and lymph nodes from PA-GFP mice, which express a photo-activatable mutant of GFP10 in all haematopoietic cells,11 were macerated through a 40 μm sieve, and CD4+ T cells were purified from released cells by negative selection. PA-GFP CD4+ T cells were incubated for 6 days in OK-DEMEM media, with 50 U recombinant IL-2, in anti-mouse CD3/CD28 antibody-coated flat-bottomed microtitre plates. Human polyclonal CD4+ T cells were isolated by negative selection (RosetteSep, StemCell Technologies) from peripheral blood leukapheresis fractions from healthy donors (New York Blood Center). Cell purity, as determined by anti-CD4/CD3 antibody labelling and flow cytometry was >95%. Freshly isolated polyclonal human CD4+ T cells were rested overnight in complete RPMI supplemented with pyruvate, and either transfected and sorted by FACS (BD FACSAria II) using a 100-nm nozzle, or incubated for 6 days in OK-DEMEM media without IL-2 for 2 hr, washed and resuspended at a density of 10⁶ cells ml⁻¹ in HBS/HSA equilibrated to 37 °C, for injection into flow chambers pre-positioned on the microscope stage and equilibrated to 37 °C using a flow chamber heating adaptor. For immunofluorescent labelling, all procedures were performed in flow-chambers. T cells attached to lipid bilayers were fixed with 2% paraformaldehyde (PFA) for 10 min at 37 °C and washed with PBS before and after, and where necessary, permeabilized for 3 min with 0.1% saponin in PBS. Samples were washed, quenched with 50 mM glycine/PBS, and blocked for 1 h with 5% BSA/PBS, before labelling with directly labelled antibodies, or unlabelled primary antibodies and appropriate fluorescently labelled secondary antibodies or Fab’ fragments (Molecular Probes). For optical–electron microscopy correlation, lipid bilayers were formed on grid substrates deposited on coverslips (described above), resulting in square fluid bilayer patches within grids, surrounded by a continuous bilayer. T cells on grids were fixed as above, and imaged using overlapping fields by TIRFM. Following optical mapping of grids, coverslips were processed for electron microscopy as described below.

Sample preparation for electron microscopy. T cells on supported lipid bilayers were fixed in flow-chambers by injecting a solution of 1% glutaraldehyde, 3% PFA, 0.3% tannic acid in 0.1 M cacoaldyrate buffer, pH 7.4 for 1 h at room temperature and processed for electron microscopy as previously described.12 Flow chambers were disassembled and fixed cells on glass coverslips removed and
post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4 for 1 h at room temperature. Samples were then dehydrated in a solution series containing 25–100% ethanol, and flat-embedded by placing upturned BEEM capsules containing liquid epoxy resin (Epon) over bilayer regions of coverslips. Capsules were stabilized with weights to ensure firm contact with coverslips, and cured at 60 °C for 48 h. Capsules were detached from coverslips by immersion in liquid nitrogen, polymerized resin blocks isolated, which contained embedded T cells and underlying bilayers on the (macroscopically) flat surface previously in contact with the coverslip. Blocks were used directly for cryosectioning to obtain views containing the entire T–cell–bilayer interface, acquired as described below, or re-embedded in Epon to obtain cross-sectional views of the T-cell–bilayer interface, in 200 nm sections collected, using a Leica UCT ultramicrotome, orthogonal to the plane of the embedded bilayer. At least three serial sections of the same cell were used to determine the plane nearest to the cell centre. To acquire sections containing the entire T-cell–bilayer interface for optical correlation, block sides were trimmed, without perturbing the block face, to a region approximately 1–2 mm², containing cell impressions and bilayer-attached T cells. The sample block was positioned on the ultramicrotome sample holder with the block face parallel to the knife-edge in the sectioning plane (Supplementary Fig. 5a), and the sample advanced to acquire a 50 nm section from the block surface. Sections were gathered on 200 hex-mesh thin bar copper grids (EMS).

Transmission electron microscopy (TEM). Conventional projection images were recorded on a Phillips CM12 transmission electron microscope, fitted with a Gatan 4k × 2.7k digital camera. Electron tomography data collection was performed in a system under the control of serialEM software. Dual tilt axis tomographic series (tilt angle ± 70°) were recorded at 200 kV in a Tecnai F20 electron microscope at the New York Structural Biology Center. Images were acquired with a 4,096 × 4,096 CCD camera and binned by a factor of 2 at 8.85 nm pixel size. Image alignment and tomographic reconstructions were obtained using the Protomo software package. Fiducial-free single-axis tomograms were combined using IMOD 5 and tomogram segmentation performed using the Amira software package. Models were constructed and rendered from segmented tomograms in IMOD 6. Tomograms of serial sections were aligned manually, guided by organelle membrane boundaries and the supported planar bilayer density. Segmentation and modelling was performed using IMOD 7. Phagophore morphology was interpreted as previously described 8.

Optical–electron microscopy correlation. Optical maps of T cells on grid bilayers acquired by TIRFM were used to screen sections containing entire grid areas by electron microscopy at low magnification. These images were then translated and rotated to match cell contours, and overlay of aligned images yielded optical–electron microscopy correlations. 9. We observed minor cell sample shrinkage in the peripheries of the cell contact interface, likely due to fixation/dehydration of electron microscopy samples, however, membrane contours within the contact centre were very well preserved, likely due to stronger adhesion, and diminished membrane fluctuations with the bilayer substrate in this region. Tomogram models of the immunological synapse centre were similarly processed to yield optical–tomogram correlations of the same cell.

Live confocal microscopy of Ca²⁺ signalling in B cells. Imaging was performed on a Zeiss LSM 510 confocal microscope. Live cell imaging was performed in FCSII flow chambers maintained at 37 °C using a heating adaptor, within an environmental chamber equilibrated to 37 °C. For Ca²⁺ imaging, lipopolysaccharide-activated B cells (incubated with or without peptides) were incubated and permeabilized with 4 mM Fluo-4 AM (invitrogen) for 20 min at 37 °C, washed and incubated in complete RPMI for 20 min. Cells were resuspended in warmed HBS/HSAs buffer at 5 × 10⁶ cells/ml, and introduced into heated flow chambers for intracellular Ca²⁺ imaging using 488/515-300 BP excitation/emission filters for Fluo-4 and 546/600 LP excitation/emission filters for detecting TCRs, labelled with AF-568-conjugated H57 Fab’ fragments. Transmitted differential interference contrast images were recorded with 457 nm illumination. 8-bit images were acquired serially using a 40× oil objective, numerical aperture 1.3, at 1,024 × 1,024 pixel resolution. Flow chambers contained bilayers with ICAM-1 alone (200 molecules per μm²), or containing isolated TCR-embedded microvesicle patches. To isolate TCR-embedded microvesicles on bilayers, TcRANCH T cells were allowed to form immunological synapse on bilayers containing MCC-I-Ek (100 molecules per μm²) and ICAM-1 (200 molecules per μm²) for 60 min, after which T cells were removed by washing flow chambers with cold HBS/HSA, that promotes de-adhesion by inactivating integrins. After screening to ensure removal of T cells, Fluo-4 loaded B cells were introduced into flow chambers, and imaged in contiguous fields after 30 min interaction with microvesicles, to survey B cell Ca²⁺ signallng, as measured by B cell Fluo-4 fluorescence intensity, in response to TCR microvesicles. For some experiments, where imaging runs were separated by long periods, necessitating independent Fluo-4 loading of separate batches of B cells, 1 μM ionomycin was introduced in flow chambers to determine maximal Ca²⁺ levels, at the end of each imaging run. This allowed correction for differences in Fluo-4 loading, expressed as individual B cell Fluo-4 fluorescence intensities/mean ionomycin induced fluorescence intensity, resulting in weighted Fluo-4 ratios.

Confocal microscopy of T–B cell conjugates. For experiments using murine T–B conjugates, lipopolysaccharide-activated B cells (incubated with or without peptides) were mixed with equal numbers of TcR AND T cells (treated with siRNA or untreated) in warmed OK-DMEM medium, and brought into contact by centrifugation at 250g for 2 min. Cells were incubated at 37 °C for 30 min, following which, media was aspirated and replaced with 2% PFA to fix cells. Cells were deposited on cleaned coverslips and permeabilized, blocked and labelled as for fluorescent labelling of TIRFM samples above, with extensive washing, by 6 exchanges with PBS, between each processing step. Following immunofluorescent labelling, coverslips were mounted on glass slides using Antifade Gold (Molecular Probes) and cured in the dark at room temperature for 24 h. Images of cell conjugates were acquired with a 63× Plan-Apochromat objective, numerical aperture 1.4.

For experiments involving human T–B conjugates, superantigen-specific CD4+ T cells were expanded from human PBMC by incubation with 100 ng ml⁻¹ of the superantigen SEE (staphylococcal enterotoxin E, Toxin Technology) in complete RPMI media. Following culture at 37 °C/5% CO₂ for 5 days, CD4+ T cells were isolated by negative selections (EasySep, StemCell Technologies), and expanded for a further 7–10 days with 10 μM 1-recombinant IL-2 (R&D Systems). Quiescent CD4+ T cells were mixed in 1:1 ratio with the human Raji B cells (ATCC CCL-86), which express HLA-DR, in the presence of 1μg/ml SEE to form superantigen induced conjugates. Cells were fixed in 3% PFA after 60 min incubation at 37 °C, permeabilized and stained with primary antibodies against TCR C/5 and an appropriate fluorochrome labelled secondary antibody. For labelling of PLC/1 phosphorylated at tyrosine 783 (Cell Signaling Technology) in addition to TCR, all incubations and labelling were performed in media/buffers containing 0.5 mM sodium orthovanadate. For selective inhibition of SYK tyrosine kinase in Raji B cells, conjugates were formed by gentle centrifugation, and incubated in complete RPMI for 30 min. Culture supernatant was then replaced, without disturbing cell pellets, with media containing 200 nM SYK inhibitor V (EMD Millipore), and cells incubated for a further 30 min at 37 °C before fixing for antibody labelling. Confocal imaging was performed as for murine T–B conjugates.

Image processing. All fluorescence images were background subtracted. Measurements from TIRFM images were made at native 14-bit depth. For optical electron microscopy correlations, images were scaled and converted to 8-bit depth. Confocal images were acquired at 8-bit depth. Measurements were performed using Meta- morph, ImageJ and Photoshop software. Presentation in figures, images were adjusted identically across related groups for brightness and contrast.

Statistical analysis. Linear regression, Student’s t-test, and one-way analysis of variance (corrected for all pairwise comparisons) were performed using Prism software. Values < 0.05 were considered statistically significant. P values > 0.05 were considered not statistically significant and denoted as NS in figures. Pearson’s correlation coefficient (Rr) was calculated from corresponding interface regions from two channels in TIRFM images using ImageJ.

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