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TCR Microclusters Pre-Exist and Contain Molecules Necessary for TCR Signal Transduction

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TCR-dependent signaling events have been observed to occur in TCR microclusters. We found that some TCR microclusters are present in unstimulated murine T cells, indicating that the mechanisms leading to microcluster formation do not require ligand binding. These pre-existing microclusters increase in absolute number following engagement by low-potency ligands. This increase is accompanied by an increase in cell spreading, with the result that the density of TCR microclusters on the surface of the T cell is not a strong function of ligand potency. In characterizing their composition, we observed a constant number of TCRs in a microcluster, constitutive exclusion of the phosphatase CD45, and preassociation with the signaling adapters linker for activation of T cells and growth factor receptor-bound protein 2. The existence of TCR microclusters prior to ligand binding in a state that is conducive for the initiation of downstream signaling could explain, in part, the rapid kinetics with which TCR signal transduction occurs.

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Abbreviations used in this article: APL, altered peptide ligand; Ch-340, 340-nm emission channel; cSMAC, central supramolecular activation cluster; EGFP, enhanced GFP; Grb2, growth factor receptor–bound protein 2; HBS/BSA, HEPES-buffered saline containing BSA; IS, immunological synapse; LAT, linker for activation of T cells; Lck, lymphocyte-specific protein tyrosine kinase; p-M, β-μ-2m, MHC, moth cytochrome c; PRST, PRS-Treen; pMHC, peptide-loaded MHC; SOS, son of sevenless; TIRF, total internal reflection fluorescence; TIRFM, TIRF microscopy.

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after ligand binding and 4 s for recruitment of Grb2 to TCR microclusters (7). TCR signaling also exhibits a digital characteristic, because presentation of a single agonist peptide by an APC leads to T cell activation and cytokine production (18, 19). The distribution of signaling molecules relative to the TCR that contributes to the speed and sensitivity of TCR signaling is not fully understood.

The relationship between ligand binding and TCR microcluster formation is not clear. If ligand binding drives microcluster formation, then reducing ligand affinity, as well as density, could modulate this process. Altered peptide ligands (APLs) contain mutations in key TCR contact residues; the result of the mutations is ligands with altered binding characteristics to the TCR that correlate with their signaling potential (20). In this study, we used APL stimulation and reduced agonist density as tools to perform a systematic analysis of the dependence of TCR microcluster formation on ligand binding. We found that some TCR microclusters exist in agonist naive cells, and their density on the cell surface does not increase in proportion to ligand density or affinity. In addition, their composition prior to ligand binding may explain the rapid kinetics with which TCR signaling is observed.

Materials and Methods

Statistical methods

Prism software was for computation of all statistical parameters. For grouped analyses, a one-way ANOVA test was performed using the Bonferroni correction, with the 95% confidence interval used for assigning significance differences.

Peptides

All peptides used in the study were synthesized commercially (American Peptide Company) and purified to >95% by reverse-phase HPLC. The sequences of the peptides used in the study were moth cytochrome c (MCC [88–103]): ANERADLIAYLKQATK, K99A: ANERADLIAYLAQATK, T102L: ANERADLIAYLKQATY, β2-microglobulin (β2m): HPPHEIQMLKGK, K5: ANERADLIAYKAAATK, ER60: GFPTIYFSPANKKL, cCYT: VNKEIQVAVVQIK, MSA: TPTILVEAARNLRGGY, HPV0: DNRMNV-HFAEAFRKRK, and α-tip: YDRNKTSPFLVGVK.

Mice and T cell activation

All mice were obtained from Taconic Farms. Additionally, all animals used in this study were maintained in a specific pathogen–free environment, and the National Institutes of Allergy and Infection Diseases Animal Care and Use Committee approved all experiments. AND TCR-transgenic RAG2−/− mice on the C57BL/6 background and RAG2−/− mice on the mixed H-2b/H-2k background were crossed to produce AND mice on the C57BL/6 background and RAG2−/− mice. Naive AND T cells from mice aged no older than 3 mo were mixed with splenocytes from mice on the mixed H-2b/H-2k background. We refer to these as AND mice. For activation, naive AND T cells were transfected using the Amaxa Mouse T Cell Nucleofector Kit (Lonza), as described in detail elsewhere (21). Briefly, 5 million AND T cells were harvested by pelleting at 75 × g for 5 min. The supernatant was removed by aspiration, and the cells were resuspended in nucleofector solution containing 5 µg plasmid DNA. Cells were transferred to an electroporation cuvette and transfected using program X-001. Following this, cells were transfected using the provided plastic transfer pipet to form quillets (a 37°C, 5% CO2 incubator for 1 h) in culture medium. Cells were rested for 4 h to allow for EGFP-tagged protein expression and used directly for imaging. Naive AND T cell transfection was carried out with the following differences: 5 µg DNA was used to transfect 10 million cells, and the cells were incubated at 30°C for 3 h following transfection. All EGFP fusion proteins used in this study were cloned between the XhoI and HindIII sites in the pEGFP-N1 expression vector (Clontech), and all cDNA used was of murine origin (Open Biosystems).

Supported lipid bilayers

Fluid lipid bilayers comprising 62.5% NI-NTA and 93.75% DOPC lipids were formed either on pinhole solution–cleaned covers glasses (Biotechs) or on silica beads (4.3 µm MicroSolv Microspheres, 10% solids; Bangs Laboratories). Unless otherwise specified, the purified extracellular domains of the following proteins were incorporated at the indicated density: histidine-tagged (on both α- and β-chain), peptide-loaded I-EK at 5 molecules/µm², histidine2-tagged ICAM-1 at 100 molecules/µm², and GPI-anchored CD80 or histidine-tagged CD80 at 100 molecules/µm². The process for preparing bilayers on both cover glasses and silica beads and quantifying the adsorbed amount of histidine-tagged molecules was described previously (22). For pMHC-trapping experiments, variants of all peptides that contained an N-terminal cysteine were synthesized (CPC Scientific) and site specifically labeled (National Institute of Allergy and Infectious Diseases Peptide Synthesis Facility) with Alexa Fluor 568–maleimide (Life Technologies). ICAM-1 was labeled with Alexa Fluor 647.

Microscopy

Simultaneous, two-color total internal reflection fluorescence (TIRF) microscopy (TIRFM) was used to study the localization of EGFP-tagged signaling proteins in TCR microclusters. Custom-designed TIRF optics that minimize chromatic effects were built around an Olympus IX71 fluorescence microscope. This method was published elsewhere in great detail (21). In this study, we briefly describe the crucial features of the system. TIRF was accomplished using a through-the-objective approach with an infinity-corrected 150×/1.45 NA TIRFM objective (Olympus). Beams from an Argon-ion and 561-nm diode-pumped solid-state laser (providing illumination of 488, 514, and 561 nm) were routed through an acusto-optic tunable filter for intensity control and, subsequently, through a single-mode fiber for delivery to the TIRF laser. Light emitted from the sample was reflected out of the right-side port of the microscope where it was split into two distinct beams (EGFP emission and Alexa Fluor 546 emission) by a dichroic mirror (Chroma) and focused by tube lenses. Finally, emission filters were placed just before each of the two identical QuantEM electron multiplying charge-coupled device cameras (Photometrics) that captured and recorded the fluorescence signal. These cameras were chosen for their sensitivity, which allowed for the observation of low levels of EGFP-tagged proteins, obviating the need for gross overexpression of signaling molecules. The combination of the 150× objective and QuantEM cameras provided a spatial sampling of 0.1 µm/pixel.

For imaging, T cells (5 × 10⁶ for agonist ligands, 10 × 10⁶ for APL ligands) were resuspended in HEPES-buffered saline containing BSA (HBS/BSA) and 10 µg/ml H57 or 13/2.3 Fab. T cells were injected into a thermostated flow chamber (Biotechs) maintained at 37°C where they were imaged for up to 1 h. To prevent photodamage, the combined laser power at the sample interface for all laser lines used was limited to 50 µW.

T cell labeling with DiO

T cells were labeled with DiO (Life Technologies) by suspending them in serum-free Advanced DMEM containing 2.0 µl DiO/1 ml at a density of 1.0 × 10⁶ cells/ml. The cells were incubated for 10 min, washed three times, and used directly for imaging.

Quantifying punctate structures

Quantifying punctate distributions of fluorophores in fluorescence microscopy images representing endosomes or clusters of cell surface proteins is always challenging. The punctate structures may have an average intensity as low as 1.2-fold over neighboring pixels that may be real cellular fluorescence signal and not background. Additionally, this basal signal may vary from cell to cell and may be nonuniform across a single cell. As a result of this complexity, threshold-based methods are insufficient to quantify such punctate distributions.

An algorithm to identify punctate structures in wide-field images of internalized low-density lipoprotein (LDL) was developed several years ago (23). This method required that images be median filtered to remove some nonuniform signal surrounding the puncta, thereby increasing the signal to noise ratio. The algorithm, in its original form, needed the following input values from the user: a threshold (T), low fraction (l), high fraction (h), step size (s), minimum area of a spot, and maximum area of a spot.
The threshold (T) is applied to the median filtered image, and all pixels less than the threshold are set to zero. After this operation, the software identifies how many spots exist. Each spot is defined as a contiguous set of pixels. This could be a single spot or many spots based on the threshold value entered. The maximum intensity pixel in each spot is identified. The software then identifies all pixels that are less than the “low fraction” times the highest-intensity pixel for each spot identified in the image and sets them to zero. A search is executed again to identify the number of spots. This operation may generate more spots or eliminate low-intensity pixels within the single spot. The software updates this information, the fraction value is incremented by the step size, and the same process is repeated. This iterative trimming process is depicted graphically (Supplemental Fig. IA) for an example image, along with the output image, in which only the punctate structures survive (Supplemental Fig. 1B). Although this process is visually must be careful to avoid losing tissues without the user. The optimal parameter set varies from cell to cell, making the analysis very tedious.

We modified the algorithm to reduce the subjective input from users and reduce the time required for analysis. Instead of asking the user to input a low fraction, a high fraction, and a step size, we asked the user to input a step size. Based on that step size, we executed the above algorithm for all combinations of low and high values. When the spots routine is executed using each of the low- and high-fraction ranges, it generates spots that have different extents of trimming. The next task was to come up with an objective criterion for choosing, at a single-spot level, the best-trimmed spot. An optimally trimmed spot in one part of the image may arise from a different set of trimming parameters than in another area.

Unique spots are generated by different trimming parameters were identified based on the maximum intensity pixel. We reasoned that an optimally trimmed spot is one whose average intensity does not change after a certain extent of trimming. We arranged each unique spot in ascending average intensity and normalized it to the highest average intensity. We then asked the user to input a cutoff parameter. The first spot above this cutoff parameter was chosen. Empirically, this parameter was chosen to be 0.83. If the cutoff parameter was 0.9, one would be choosing a more-trimmed spot; if it were <0.83, one would be choosing a less-trimmed spot.

In this modified algorithm, the minimum spot area, maximum spot area, and threshold are still determined by the user.

Measurement of calcium fluxes

For the measurement of calcium fluxes, preactivated AND T cells were loaded with the calcium sensitive dye Fura-2 AM (Life Technologies). AND T cells were pelleted at 80 × g, washed once with 10 ml serum-free Advanced DMEM (Life Technologies), and resuspended in 1 ml room temperature serum-free Advanced DMEM containing 2 μM Fura-2. Cells were incubated at room temperature for 25 min in the dark. After this, 5 ml prewarmed Advanced DMEM containing 2.5% serum was added to the cells, and the cells were incubated for an additional 30 min at 37°C. The cells were pelleted at 80 × g and used directly for imaging with a 4×, 1.35 NA objective (Olympus; UAP040XOI3/340); fluorescence emission was recorded for 340- and 380-nm excitation. IRM and ICAM-1 images also were collected so that cells adhering to the bilayer could be identified. As a control to determine the maximum calcium flux, the cells were subjected at the end of the experiment to treatment with 2 μg/ml ionomycin in HBS/BSA containing 2 mM MgCl₂ and 20 mM CaCl₂. Finally, as a control to measure the baseline Fura-2 fluorescence emission, the cells were subjected to treatment with 2 mM EGTA in calcium-free HBS/BSA containing 3 mM MgCl₂.

Cell segmentation and tracking for calcium flux analysis

Single-cell calcium flux analysis was accomplished by first segmenting the cells in the 340-nm emission channel (Ch-340) and then tracking these binary masks corresponding to the cells. In the segmentation phase, individual Ch-340 frames of the two-dimensional time series data were preprocessed to enhance circular structures (cells) using a band-pass filter. The foreground objects from the preprocessed Ch-340 frame were identified using Kittler and Illingworth’s Minimum-Error algorithm. However, this leads to oversegmentation, especially when cells are closely packed. Hence, watershed, a morphological-based segmentation, was used to break down large foreground objects into individual cells. Postsegmentation, a size (<180 pixels and >2500 pixels) and shape (circularity [0.6, 1.0]) filtering removes small islands and large foreground objects (if any). All segmented objects at the edges of the frame are also ignored. These image-processing operations were implemented as an ImageJ macro with batch-processing capability.

Once the binary masks for the cells in the Ch-340 were identified, a first-order autoregression algorithm (24) implemented as MATLAB script (MathWorks) was used for automatic object tracking in the two-dimensional time series data. Next, the MATLAB script applied a fully corrected (rolling-ball algorithm with radius of 20 pixels) 340- and 380-nm emission channels, raw IRM and ICAM-1, and binary masks of the cells from the segmentation step as a multichannel two-dimensional time dataset into Imaris (Andor/Bitplane), using XTension programming interface. The tracking information from the autoregression algorithm was also exported into Imaris as output objects within this file. Imaris was used for manual/visual selection of cell tracks that made contact with the bilayer (visible in the IRM and ICAM-1 channel). The user-selected tracks were exported to a Microsoft Excel workbook. For each selected track, Imaris was configured to export the mean intensity in the 340- and 380-nm emission channels so that the ratio of the two values could be calculated.

Western blotting

For cell-stimulation experiments, 1 μl silica bead suspension was used for 0.2 × 10⁶ T cells. This corresponds to 10 silica beads/cell. For LAT immunoblots, 5 million cells/concentration was stimulated, followed by lysis in a buffer containing 0.5% Brij-96, 150 mM NaCl, 20 mM Tris-HCl (pH 9), 0.2% EDTA, 1 mM PMSF, 1 mM NaVO₃, 1× PhosStop phosphatase inhibitors, and 1× Complete Mini protease inhibitors (Roche) for 30 min on ice. Following this, cellular debris was pelleted at 14,000 rpm at 4°C, and the cleared supernatants were transferred to new tubes. Finally, SDS loading dye was added to the lysates, followed by boiling for 6 min with intermittent vortexing. For ERK immunoblots, the process was the same except that 1% Nonidet P-40 was used as the detergent, and only 2 million cells were stimulated per condition. Lysates were run on 4–20% SDS/PAGE gradient gels (Bio-Rad) using a current of 25 mA/gel, and proteins were transferred to nitrocellulose membranes using the iBlot semidry transfer system (Life Technologies). Membranes were blocked with 5% blotting-grade milk (Bio-Rad) in PBS–TWEEN (PBST) for 1 h at room temperature, followed by incubation with primary Abs in PBST overnight at 4°C with gentle mixing. After washing with PBST, membranes were probed with HRP-labeled anti-rabbit IgG (Cell Signaling; #7074) for 1 h at room temperature. p-LAT (Y191; Abbac; #ab-59197) and LAT (Cell Signaling; #9166) were detected using photographic film (BioMax XAR; Kodak). ERK and p-ERK (Cell Signaling; #4692 and #9102, respectively) were detected using a charge-coupled device–based chemiluminescence detection system (FluoChemQ; Alpha Innotech).

Generation of Fab Ab fragments

Fab fragments of H57, against murine TCRβ-chain, and I3/2.3, against murine CD45, Abs were generated as follows. Affinity-purified Abs were concentrated to 2 mg/ml in PBS. PBS buffer was exchanged by dialysis against 50 mM citrate buffer at pH 5.5 containing 50 mM NaCl and 25 mM cysteine (added just prior to digestion). For digestion of the Abs, a mixture of 2 mg/ml papain (Sigma), 2 mM EDTA, 1 mM DTT, and 40 mM cysteine was prepared in 100 mM Tris-HCl buffer (pH 8). Following activation of the papain-containing solution at 37°C for 10 min, 5 μl activated papain solution was added per milligram of Ab to the Ab-containing solution and allowed to digest for 3 h at 37°C. The reaction was quenched by the addition of 5 mM iodoacetamide.

For H57, the Fab,Fc, and undigested whole Abs were separated by ion-exchange chromatography using DEAE Sephrose beads (GE Healthcare). After dialysis against 10 mM Tris buffer at pH 8, the digestion mixture was bound to the ion-exchange media. Separation of the fragments was accomplished by washing the column with 10 mM Tris buffer at pH 8, containing 50, 100, 200, 400, and 1000 mM NaCl. The Fab fragment elutes in the 50-mM NaCl wash, whole Ab elutes at 100 mM NaCl, and the Fc fragment elutes at 200 mM NaCl. Separation was verified using SDS/PAGE. For I3/2.3, attempts to separate Fab, Fc, and undigested whole Abs by ion-exchange chromatography were unsuccessful. In lieu of this, Fab and Fc fragments were separated from whole Ab by size-exclusion chromatography and used directly for cell labeling. Fab fragments for both Abs were labeled with Alexa Fluor 488 using standard methods and stored at 4°C in PBS at a concentration of 1 mg/ml. After separation, the digestion products were run on an SDS-PAGE gel to ensure that no F(ab′)₂ fragments were present.
Results

APL potency hierarchy for AND TCR

To study the relationship between ligand binding and TCR microclusters, we wanted to establish a range of pMHC-TCR potencies (as defined by their ability to cause calcium fluxes and ERK activation at a fixed Ag dose). We chose to compare the agonist peptide MCC (88–103), presented by the MHC class II molecule I-Ek, and two APLs of the AND TCR, K99A and T102L. Additionally, we included an I-Ek–binding self-peptide derived from β2m (25, 26) as a negative control, which does not act as a coagonist for the AND receptor (Supplemental Fig. 2). Our experimental approach involved exposure of T cells to coverslips coated with fluid lipid bilayers in which agonist or APL-loaded pMHCs, the costimulatory molecule CD80, and ICAM-1 were linked via histidine tags to Ni-NTA head-group bearing lipids. T cells interacting with the bilayer were imaged using TIRFM.

The functional characteristics of these APLs have been well studied (27–29), and a hierarchy of potencies has been established: MCC > K99A in the presence of CD80 > K99A > T102L > β2m. K99A is a costimulation-dependent agonist capable of inducing proliferation (27) and inducing calcium fluxes only in the presence of CD80 costimulation, and T102L is an APL that does not elicit proliferation or calcium fluxes but causes in vivo tolerance (29).

We ensured that this hierarchy of potencies was reproducible under our experimental conditions by measuring calcium fluxes and ERK activation in preactivated AND T cells in response to agonist and APL stimulation at a dose of 5 pMHC molecules/μm² (Fig. 1 A, 1B). To ensure that the stimulation conditions used for assaying ERK activation were identical to those used for calcium flux measurements, we prepared silica beads coated with fluid lipid bilayers presenting either agonist or APL-loaded pMHC, CD80, and ICAM-1 to use as “artificial APCs” for T cell stimulation and performed Western blot analysis to detect phosphorylation of ERK. Stimulation with both MCC and K99A in the presence of CD80 produced calcium fluxes, whereas stimulation with K99A in the absence of CD80 and T102L did not. Similarly, T102L stimulation was not sufficient to activate ERK over background levels. The average fold increase in ERK activation for two experiments is presented in Fig. 1C. The hierarchy of APL potency mirrored the ability of a particular ligand to stably interact with the AND TCR within the immunological synapse, because AND T cells trapped each of the APL-loaded pMHCs, with the exception of the negative-control peptide β2m (Fig. 2).

The TCR content of a microcluster is invariant, and TCR microcluster density is not a strong function of pMHC potency

Having established the relative potencies of APL-loaded I-Ek, we next wished to examine the characteristics of the IS and TCR microclusters generated in response to each APL. Representative images of synapses formed by preactivated AND T cells in response to each pMHC ligand, at a density of five pMHC molecules/μm², in the presence of 100 molecules/μm² of both CD80 and ICAM-1, are shown in Fig. 3A. Only exposure to the agonist peptide MCC led to the formation of a cSMAC, as reported previously (27, 30).

We wished to determine whether ligand binding modulated the characteristics of TCR microclusters. TCR microclusters were readily detectable in all conditions tested, including on the membrane of T cells interacting with bilayers containing β2m–I-Ek, even though AND T cells do not trap this ligand. To ensure that the bright accumulations of TCR observed in the case of β2m–I-Ek stimulation actually represented TCR microclusters and not regions of the membrane simply coming into close proximity to the lipid bilayer, we costained AND T cells with Fab fragments against TCRβ and the membrane dye, DiO. Consistent with previous findings (4), DiO is not enriched in TCR microclusters present on the membranes of AND T cells being stimulated with MCC–I-Ek, but it does accumulate within the cSMAC after the formation of a mature IS. In the case of β2m stimulation, there is no observable enrichment of DiO within areas of TCR accumulation, confirming that these structures are indeed TCR microclusters (Supplemental Fig. 3).

To determine the differences between TCR microclusters present in preactivated T cells being stimulated by the panel of APLs, we graphically identified TCR microclusters for all stimulation conditions using an automated segmentation algorithm (Materials and Methods, Supplemental Fig. 1A, 1B) and quantified their absolute numbers and characteristics. T cells stimulated with T102L accumulated more TCR microclusters on average (17) than did cells stimulated with the control self-peptide β2m (10) (Fig. 3B). This trend continued as the potency of the peptide increased, with K99A, irrespective of the presence of CD80, and MCC accumulating an average of 22 and 35 TCR microclusters, respectively. Because cell spreading became more robust as the potency of the
pMHC being presented increased (Fig. 3C), it was important to normalize the absolute number of TCR microclusters to the spread area of the cell to determine whether the observed increase in absolute microcluster number was simply due to our ability to observe more total membrane area. After normalization, we found that the average number of TCR microclusters/unit of contact area (TCR microcluster density) increased only slightly, from 0.2 to 0.35 microclusters/μm², when comparing cells stimulated with β2m with those stimulated with APLs (Fig. 3D1). A significant difference in microcluster density also was observed between cells stimulated with MCC or T102L; however, no other pair-wise differences in average microcluster densities were detected at this Ag dose (5 molecules/μm²) (Fig. 3D2). We also wished to know whether the TCR microcluster density for each ligand varied with time or was a true constant. To determine this, we plotted the observed TCR microcluster density from the cells in Fig. 3D1, as a function of the time since they were injected into the flow cell (Supplemental Fig. 4); we observed that the microcluster density fluctuated around the mean of the distribution. This indicated that the microcluster density was a constant and did

FIGURE 2. APL-loaded pMHC trapping by TCR microclusters. (A) Agonist or APL-loaded I-Ek (5 molecules/μm²) loaded with Alexa Fluor 568–labeled peptides were presented to preactivated AND T cells along with CD80 (100 molecules/μm²) and ICAM-1 (100 molecules/μm²) on fluid lipid bilayers. For each pMHC, two representative cells are depicted. For each set of representative images, two fluorescence intensity scales are depicted. “MCC bright” and “T102L bright” refer to a fluorescence intensity scale at which clusters of the respective pMHC are readily apparent, allowing for comparison of relative intensity differences between the various pMHC clusters. Arrowheads identify especially bright clusters of T102L-I-Ek. Scale bar, 2.5 μm. (B) The efficiency of trapping for each pMHC was calculated as the ratio of pMHC fluorescence intensity beneath the bright-field image of the cell/fluorescence intensity in regions in the same image where no cell was present. The solid horizontal line at a pMHC trapping of 1.0 indicates the condition when the pMHC intensities below and outside the confines of the cell are equivalent. Error bars represent the mean ± SEM. A minimum of 20 individual cells was quantified for each condition. Data are representative of two independent experiments. For trapping measurements, the fold fluorescence increases are reported for individual cells that had been in contact with the bilayer for up to 1 h. **p < 0.001, ***p < 0.0001, one-way ANOVA with Bonferroni correction. n.s., not significant.
FIGURE 3. IS and microcluster formation in response to APLs. (A) Agonist or APL-loaded I-E\(^{k}\) (5 molecules/\(\mu m^2\)) were presented to preactivated AND T cells stained with Alexa Fluor 546–labeled H57 Fab fragments along with CD80 and Alexa Fluor 647–labeled ICAM-1 on fluid lipid bilayers. Scale bar, 2.5 \(\mu m\). (B) Total TCR microcluster counts resulting from stimulation of AND T cells with the pMHC ligands indicated in (A). For MCC-I-E\(^{k}\), the cSMAC was excluded from the analysis so that only the number of peripheral microclusters was quantified. (C) Graphs of the total membrane area visible for the cells in (B) in the TIRF plane for each pMHC ligand. Relative frequency is the percentage of the total number of cells presenting with the indicated area. Each point represents the center of a bin of 2500 pixels. Lines serve as guides for the eye to aid in distinguishing the distributions. Cell contact areas were determined by H57 Fab staining. (D1) TCR microcluster counts at a dose of 5 molecules/\(\mu m^2\) from (B) normalized to the interfacial area of the T cell (\(A\)). MCC at a dose of 0.05 molecules/\(\mu m^2\) (\(C\)), and T102L at a dose of 500 molecules/\(\mu m^2\) (\(Q\)). For clarity, the dose of pMHC ligand is also listed in parentheses (molecules/\(\mu m^2\)) for each stimulation condition. (D2) Statistical significance of pair-wise comparisons from (D1). (Figure legend continues)
not vary over the period of time during which we monitored the cells.

To further probe the effects of Ag dose on TCR microcluster density, we next presented AND T cells with both a limiting dose of agonist ligand (0.05 molecules/μm²), corresponding to approximately five pMHC ligands/cell, and a supraphysiological dose of the low-potency ligand T102L (500 molecules/μm²) (Fig. 3D1). In response to the limiting dose of agonist ligand, the TCR microcluster density decreased to the same level as cells stimulated with β2m. However, stimulation with the very high dose of T102L failed to increase the microcluster density above levels achieved with a 100-fold lower dose of the ligand.

Additionally, we quantified the fluorescence intensities of individual TCR microclusters for each stimulation condition (Fig. 3E). With the exception of a statistically significant 30% increase in microcluster intensity between β2m and both MCC and T102L, we found that the fluorescence intensities of the microclusters present on the membranes of cells stimulated with any of the other ligands were statistically indistinguishable. To ensure that microcluster intensity differences were reflective of TCR content, rather than changes in microcluster size that were a function of ligand potency, we plotted frequency distributions of microcluster sizes for each of the ligands and found them to be identical (Fig. 3F). With the exception of the small difference in TCR content between microclusters observed under the stimulatory versus the nonstimulatory control, these results suggest that engaging ligands of different affinities do not change the number of TCRs within a microcluster.

To rule out the possibility that the presence of microclusters in cells stimulated with the negative-control peptide were the result of prior in vitro activation, we next imaged naive AND and polyclonal T cells interacting with bilayers containing only CD80 and ICAM-1 (Fig. 4) and again observed the presence of TCR microclusters. These results further confirmed that TCR microclusters pre-exist in the absence of prior agonist pMHC engagement. CD28–CD80 interactions also were shown to localize to TCR microclusters (6). To rule out that these interactions may have contributed to the formation of microclusters, we imaged cells on substrates containing only ICAM-1 and found that microclusters could still be observed.

**Molecular composition of pre-existing TCR microclusters**

We established that TCR microclusters were able to trap APL-loaded pMHC ligands and that the quantitative receptor representation is not a function of APL potency. Agonist-induced TCR microclusters were shown to exclude the phosphatase CD45 and be enriched in signaling molecules implicated downstream of TCR engagement, such as ZAP70 (3, 5), LAT (3), Grb2 (3, 7), phospholipase Cγ1 (31), the SH2 domain-containing leukocyte protein of 76 kDa (3, 5), and others. We next wished to examine the composition of the ligand-independent, pre-existing TCR microclusters. We first examined whether such TCR microclusters excluded the phosphatase CD45, because it was hypothesized to be excluded from sites of TCR–pMHC interaction, according to the kinetic segregation model. To determine whether CD45 was being excluded from TCR microclusters upon stimulation with APLs, we costained AND T cells with Fab Ab fragments against both TCRβ and CD45 and imaged them interacting with fluid lipid bilayers containing agonist or APL pMHC, along with CD80 and ICAM-1, CD80 and ICAM-1, or ICAM-1 alone (Fig. 4). We found that CD45 was excluded from TCR microclusters under all stimulation conditions, including in the absence of TCR ligation. This finding extended to both naive AND and naive polyclonal T cells stimulated with CD80 and ICAM-1, indicating that TCR-agonist pMHC engagement is not required for the exclusion of CD45 from TCR microclusters.

We next examined the distribution of the adapter molecule LAT with respect to TCR microclusters, because this adapter was shown to interact with TCR microclusters only after recognition of an activating ligand (13). We imaged preactivated, LAT–EGFP–expressing AND T cells interacting with agonist and APL-presenting fluid lipid bilayers containing CD80 and ICAM-1 (Fig. 5A). We observed that LAT was present in TCR microclusters in all stimulation conditions that we examined. Further, we quantified the number of both TCR and LAT–EGFP microclusters using the segmentation algorithm described earlier (Fig. 5B). We found that the average numbers of TCR and LAT–EGFP microclusters were roughly equivalent for each respective stimulation condition, and both the number of TCR and LAT–EGFP microclusters otherwise increased as the potency of the ligand increased. In each case, the signal from TCRβ and LAT–EGFP strongly colocalized, and the degree of colocalization did not vary among pMHC ligands (Fig. 5C, Supplemental Videos 1–5). Additionally, we quantified the relative intensity of the LAT–EGFP microclusters for each stimulation condition (Fig. 5D). Because the amount of EGFP signal recorded using TIRFM at the T cell–bilayer interface is directly proportional to the expression level of the EGFP-tagged protein (Supplemental Fig. 1C), we normalized the raw intensity of each LAT–EGFP cluster to the expression level of LAT–EGFP in the T cell. In this analysis, we found that, compared with ligands that do not cause detectable calcium flux, the intensity of LAT–EGFP clusters significantly decreased on T cells stimulated with ligands that do cause a calcium flux. This result is consistent with the c-Cbl–mediated endocytosis of LAT upon T cell activation reported by Balagopalan et al. (32).

We next expressed LAT–EGFP in naive AND T cells and incubated them with lipid bilayers containing β2m–I-Ek, CD80, and ICAM-1. We observed TCR microclusters with which LAT strongly colocalized (Fig. 5A). Additionally, we quantified the number of TCR and LAT–EGFP microclusters in these naive cells (Fig. 5E), as well as their colocalization with one another (Fig. 5F), and found that the results were highly similar to activated cells stimulated with β2m–I-Ek. Taken together, these results indicate that the partitioning of LAT into pre-existing TCR microclusters is a constitutive property of agonist-naive T cells and not the result of in vitro activation.

Because LAT was present in TCR microclusters, irrespective of pMHC potency, we sought to determine in response to which ligands LAT was being activated. To accomplish this, we prepared artificial APCs presenting agonist or APL-loaded pMHC, CD80, and ICAM-1 and performed Western blots to detect phosphorylation of LAT at position 191 (Y191) (Fig. 5G). As expected, LAT was robustly phosphorylated in response to MCC stimulation.

**Supplemental Methods**

**Supplemental Figure 1**

**E**. Intensities of individual microclusters from (B). Different conditions are grouped into the indicated size range on the x-axis. The y-axis is the percentage of the total number of TCR microclusters presenting with the indicated size. Each point represents the center of a bin of five pixels. Lines serve as guides for the eye to aid in distinguishing the distributions. Test. For all panels, error bars represent the mean ± SEM. Data in (B)–(F) are pooled from cells that had been in contact with the bilayer substrate for up to 15 min. Data are representative of two independent experiments. *p < 0.01, **p < 0.001, ***p < 0.0001, n.s., not significant.
K99A stimulation in the presence of CD80 caused a small amount of LAT phosphorylation, approximately one order of magnitude less than that caused by stimulation with MCC, consistent with its ability to elicit a small calcium flux. We were unable to detect LAT phosphorylation at position 191 in response to all other ligands. The average fold increase in LAT activation for two experiments is presented in Fig. 5H. These results demonstrate that LAT constitutively partitions into TCR microclusters independently of its activation status.

Grb2, an adapter protein containing SH2 and SH3 domains, binds phosphorylated residues on LAT via its SH2 domain. Grb2 bound to p-LAT can facilitate ERK activation through its interaction with son of sevenless (SOS). In addition to functioning as a RasGEF, SOS1 was shown to function as a scaffold protein and
cause oligomerization of LAT (33). This scaffold function of SOS1 requires its interaction with Grb2. Because we observed reduced TCR-induced ERK activation in response to APL stimulation (Fig. 1B), we questioned whether this was a result of reduced Grb2 binding to LAT within TCR microclusters. To address this, we determined the distribution of Grb2 relative to TCR microclusters in response to APLs that did not induce LAT phosphorylation. We transfected preactivated AND T cells with a Grb2-EGFP construct

FIGURE 5. LAT recruitment to TCR microclusters and activation in response to APL stimulation. (A) Agonist or APL-loaded I-Ek (five molecules/μm²) was presented to preactivated or naive LAT–EGFP–transfected AND T cells stained with Alexa Fluor 546-labeled H57 Fab fragments along with CD80 and ICAM-1 on fluid lipid bilayers. (B) Total TCR microcluster and LAT–EGFP cluster counts resulting from stimulation of AND T cells, as described in (A). For MCC–I-Ek, the cSMAC was excluded from the analysis so that only the number of peripheral microclusters was quantified. (C) Colocalization of TCR and LAT–EGFP microclusters from (B). Colocalization was calculated as the percentage of TCR microclusters overlapping in area with a LAT–EGFP cluster by ≥40%. (D) Fluorescence intensity of individual clusters of LAT–EGFP normalized to the total expression level of LAT–EGFP in each cell resulting from stimulation of AND T cells, as described in (A). (E) Total TCR microcluster and LAT–EGFP cluster counts resulting from stimulation of LAT–EGFP–transfected naive AND T cells with β2m–I-Ek. (F) Colocalization of TCR and LAT–EGFP microclusters from (E). For data in (B)–(F), error bars represent the mean ± SEM, and data are pooled from cells that had been in contact with the bilayer substrate for up to 1 h. (G) Fluid lipid bilayers containing agonist or APL-loaded I-Ek (five molecules/μm²), CD80, and ICAM-1 were prepared on silica beads and used to stimulate preactivated AND T cells for 3 min. The resulting blots were probed with an Ab against LAT phosphorylated at Y191. Ratio of the intensity of p-LAT/total LAT is listed below each lane. The blot was cropped to show the band of interest. (H) Average fold change in LAT activation with respect to cells exposed to bilayers containing only ICAM-1 and CD80 for two separate experiments, with error bars representing mean ± SD. Data are representative of two independent experiments. See also Supplemental Videos 1–5. Scale bar, 2.5 μm. ***p < 0.0001, one-way ANOVA.
and imaged them as described (Fig. 6A). Like LAT, Grb2 was present in TCR microclusters in response to stimulation by all ligands. As before, we counted the total number of microclusters of TCR and Grb2–EGFP and again found that, for each stimulation condition, the average numbers of TCR and Grb2–EGFP microclusters were roughly equivalent (Fig. 6B) and increased as the potency of the ligand increased. In each case, the signal from TCR and Grb2–EGFP strongly colocalized with one another (Fig. 6C, Supplemental Videos 6–10). Further, we quantified the fluorescence intensity of the Grb2–EGFP microclusters and found that, with the exception of an order of magnitude increase in response to MCC stimulation, the amount of Grb2 present within a microcluster was not a function of ligand potency (Fig. 6D). Again, similar to CD45 exclusion and LAT partitioning, a basal level of association of Grb2–EGFP with TCR microclusters was independent of T cell activation. In such situations when LAT is not phosphorylated at residues necessary for Grb2 recruitment, Grb2 could be bound via its SH2 domain to several proteins within a TCR microcluster, because it was shown to interact with proteins, such as CD28 (34) and TCRζ (35), via its SH2 domain. Alternatively, Grb2 could bind to proline-rich regions in other proteins within the microcluster via SH3 domain interactions. To allay concerns relating to artifacts of prior activation of the T cell, we expressed Grb2–EGFP in naive AND T cells and found a constitutive localization of Grb2–EGFP with pre-existing TCR microclusters on these agonist-naive cells (Fig. 6A). Additionally, we quantified the number of TCR and Grb2–EGFP microclusters in these naive cells (Fig. 6E) and their colocalization with one another (Fig. 6F) and found that the results were highly similar to activated cells stimulated with β2m–I-Eκ. Thus, Grb2 is contained within ligand independent, pre-existing TCR microclusters, albeit at levels 10-fold lower than found in TCR microclusters engaging agonist TCR ligands.

In conclusion, we found that some TCR microclusters pre-exist in unstimulated T cells. Such microclusters excluded the phosphatase

FIGURE 6. Grb2 recruitment to TCR microclusters in response to APL stimulation. (A) Agonist or APL-loaded I-Eκ (five molecules/μm²) were presented to preactivated or naive Grb2–EGFP–transfected AND T cells stained with Alexa Fluor 546–labeled H57 Fab fragments along with CD80 and ICAM-1 on fluid lipid bilayers. (B) Total TCR microcluster and Grb2–EGFP cluster counts resulting from stimulation of AND T cells as described in (A). For MCC–I-Eκ, the cSMAC was excluded from the analysis so that only the number of peripheral microclusters was quantified. (C) Colocalization of TCR and Grb2–EGFP microclusters from (B). Colocalization was calculated as the percentage of TCR microclusters overlapping in area with a Grb2–EGFP cluster by ±40%. (D) Fluorescence intensity of individual clusters of Grb2–EGFP normalized to the total expression level of Grb2–EGFP in each cell resulting from stimulation of AND T cells as described in (A). Unmarked comparisons were not statistically significant. (E) Total TCR microcluster and Grb2–EGFP cluster counts resulting from stimulation of Grb2–EGFP–transfected naive AND T cells with β2m–I-Eκ. (F) Colocalization of TCR and Grb2–EGFP microclusters from (E). For all panels, error bars represent mean ± SEM. For (B)–(F), data are pooled from cells that had been in contact with the bilayer substrate for up to 1 h. Data are representative of two independent experiments. See also Supplemental Videos 6–10. Scale bar, 2.5 μm. ***p < 0.0001, one-way ANOVA.
CD45 and were enriched in the signaling adapters LAT and Grb2. The pre-existence of some TCR microclusters at the basal state enriched with signaling molecules may explain the rapid kinetics with which TCR signaling occurs.

Discussion
It is well appreciated that TCR microclusters are sites for active TCR signaling, but controversy still exists with regard to how and when they are formed (36). Several lines of evidence now exist that indicate that TCR aggregates of some sort pre-exist (8, 11–13). We showed previously that some TCR microclusters could be detected in unstimulated cells, but a full characterization was not carried out (4). In live cells, our data show that TCR microclusters are present on T cells never exposed to agonist ligand. These pre-existing microclusters increase in absolute number as the potency of pMHC ligands increases at a fixed Ag dose; however, this increase in absolute number with increasing ligand potency seems to be a consequence of increased cell spreading, which exposes more total membrane area. As a consequence, the density of TCR microclusters present on the membrane is not a strong function of Ag potency, either achieved through change in the Ag dose or affinity. Interestingly, the size of a microcluster and the number of TCRs contained within it are also not a function of the pMHC potency. Although we detected a slightly lower intensity for microclusters observed upon stimulation with β2-m, we attribute this result to this ligand’s inability to stably engage the microclusters and the probable dynamic nature of the pre-existing clusters before they are stabilized through interactions with more potent ligands. This well-defined composition explains our observation that pMHC ligands of very low potency are excluded from microclusters and that a single perturbation is unable to stably engage the microclusters, because binding and rebinding of individual pMHCs within the same microcluster are possible because of the high local concentration of TCR.

Similar to the invariant nature of TCR content within a microcluster, we observed that TCR microclusters present on T cells in the absence of Ag stimulation constitutively excluded the phosphatase CD45. This finding is not consistent with the kinetic segregation model of T cell signaling that holds that the large and heavily glycosylated extracellular domain of CD45 causes its exclusion from TCR microclusters, where long-lived binding of the TCR with pMHCs causes the formation of tight junctions between the T cell membrane and APC. T cells expressing chimeric CD45 molecules in which the ectodomain was replaced with the shorter ectodomain of either CD2 or Thy 1 showed dramatic defects in the activation of NFAT (37); further, recent imaging studies showed that CD45 mutants with truncated ectodomains fail to be excluded from TCR microclusters (38). We also considered that the formation of CD28–CD80 interactions in our system could be driving the exclusion of CD45 from TCR microclusters, because CD28 colocalizes with TCR microclusters (6), and the interacting pair could create a tight membrane contact capable of stERICALLY EXCLUDING CD45. We ruled out this possibility by demonstrating that CD45 is still excluded from microclusters on cells interacting with bilayers containing only ICAM-1.

We also found that pre-existing TCR microclusters contained both LAT and Grb2. Sherman et al. (12) reported the existence of protein nanostructures at the basal state in which LAT and Grb2 do not colocalize. Although we found Grb2 and LAT together in the microcluster at the basal state, the optical resolution of our technique does not allow us to make conclusions about their physical association. Given that our data indicate that LAT is not phosphorylated at the residues necessary for Grb2 association, it is likely that Grb2 is interacting with some other proteins that are present in the microcluster at the basal state, as mentioned previously. Importantly, we found that the presence of LAT and Grb2 in pre-existing TCR microclusters was not an artifact of previous T cell activation because we observed both in microclusters on naive and T cells.

Interestingly, the signaling behavior of T cells in response to K99A stands out from the other ligands studied with respect to its dependence on costimulation by CD80. Although there is virtually no variation in the amount pMHC trapping, the total number of microclusters generated, or the amount of ERK activated, the amount of cell spreading, calcium flux, and LAT activation are clearly affected when CD80 is not present. When CD80 is present in the bilayer, we believe that CD28 may act to bring the lymphocyte-specific protein tyrosine kinase (Lck) into the microcluster to initiate downstream signaling, because it was shown to act as a scaffold for Lck (39). When this happens, Lck-dependent phosphorylation events lead to more LAT phosphorylation and additional signals that lead to generation of calcium fluxes.

The mechanisms that drive the formation of the pre-existing microclusters that we observed remain elusive, but it is clear that recognition of agonist ligands is not required for their formation because we showed that TCR microclusters pre-exist on the surface of naive T cells that have never seen an agonist ligand. It was demonstrated that depolymerizing the actin cytoskeleton prevents the formation of TCR microclusters (4, 40), but other investigators argued that actin is necessary for microcluster maintenance but not formation (13). It is likely, then, that a TCR microcluster is a collection of steady-state nanoclusters pre-associated with signaling molecules corralled by actin structures. Another possibility is that TCR microclusters are some form of surface-associated endocytic structures. We think that a combination of cell biological phenomena leads to the formation of these pre-existing microclusters and that a single perturbation is unlikely to reveal the precise mechanism of their formation. It is to be noted that there are differences in the composition and physical properties of TCR microclusters observed in response to agonist ligands and those that exist in the unstimulated state. The amounts of Grb2 (this article) and ZAP70 (K. Padhan, T. Crites, A. Knauf, and R. Varma, manuscript in preparation) are much higher in TCR microclusters engaging agonist ligands. Microclusters observed in response to APLs exhibit sensitivity to actin depolymerization that is not seen in response to agonist stimulation (30).

Taken together, our results indicate that pre-existing TCR microclusters are specialized structures with a well-defined composition. Biochemical reactions occurring in the plasma membrane are often diffusion limited. This preorganized state, in which positive regulators of TCR signal transduction are preassociated but negative regulators are excluded, could compensate for the slow diffusion of interacting components and could facilitate the ability of the TCR to rapidly signal when a high-potency pMHC is encountered (7).

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Information

A

Original Image

Iterative Trimming

B

Original Image

Spots Program Output

Overlay

C

Wide Field Intensity

2.0×10^8

1.5×10^8

1.0×10^8

5.0×10^7

0.0

0

2×10^7

4×10^7

6×10^7

8×10^7

TIRF Intensity
Supplementary Figure 1: Identification of Punctate Structures Using Spots Analysis and TIRF Intensity Linearly Correlates with Protein Expression Level

(A) An example image of Grb2-EGFP is subjected to iterative trimming to identify Grb2-EGFP microclusters. (B) The original Grb2-EGFP image is overlaid with the final spots program output image following trimming. Scale bar = 2.5 µm (C) The wide field intensity of several T cells expressing LAT-EGFP, a measure of protein expression, is plotted against the total TIRF intensity of each cell. As the relationship is highly linear, \( r^2 = 0.92 \), the wide field intensity is used as a normalizing factor for comparing the intensity of microclusters of EGFP-tagged proteins between cells expressing the protein at varying levels.
Supplementary Figure 2: $\beta$-2m-I-E$k$ is not a Coagonist pMHC for the AND TCR

Several I-E$k$ binding endogenous peptides were tested for their ability to act as coagonists for the AND TCR. Co-activation scores, represented as the percentage of cell fluxing calcium, were assayed by loading CHO-GPI-I-E$k$ cells with 0.1 µM K5 peptide with or without 10 µM endogenous peptide and measuring the Ca$^{2+}$ response of AND T cells by labeling with fura-2.
Supplementary Figure 3: TCR Microclusters are not an Artifact of TIRF

Microscopy MCC or β-2m-loaded I-E^k (5 molecules/µm^2) was presented to preactivated AND T cells costained with AlexaFluor 546-labeled H57 Fab and DiO fragments along with CD80 and ICAM-1 on fluid lipid bilayers. For MCC stimulation, images of the cell are presented both before and after cSMAC formation. In the case of both MCC and β-2m stimulation, no accumulation of DiO is present in areas of TCRβ accumulation. Scale bar = 2.5 µm
Supplementary Figure 4: TCR Microcluster Density Does Not Vary in Time

The microcluster density resulting from stimulation with 5 molecules/µm² of the indicated
ligands for the cells from Figure 3D1 are plotted according to time since the cells were in injected into the flowcell. Connecting lines serve as guides for the eye. The dashed lines on each plot represents the mean of the distribution from Figure 3D1. Error bars represent ± SD.
Legends for Supplementary Movies

Supplementary Movie 1 (Related to Figure 5): LAT Colocalizes with TCR

Microclusters in Response to Stimulation with MCC-I-E^k Preactivated AND T cells were incubated with fluid lipid bilayers containing MCC-I-E^k, CD80 and ICAM-1. Images of LAT-EGFP and TCRβ were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 μm

Supplementary Movie 2 (Related to Figure 5): LAT Colocalizes with TCR

Microclusters in Response to Stimulation with K99A-I-E^k in the Presence of CD80 Preactivated AND T cells were incubated with fluid lipid bilayers containing K99A-I-E^k, CD80 and ICAM-1. Images of LAT-EGFP and TCRβ were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 μm

Supplementary Movie 3 (Related to Figure 5): LAT Colocalizes with TCR

Microclusters in Response to Stimulation with K99A-I-E^k in the Absence of CD80 Preactivated AND T cells were incubated with fluid lipid bilayers containing K99A-I-E^k and ICAM-1. Images of LAT-EGFP and TCRβ were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 μm

Supplementary Movie 4 (Related to Figure 5): LAT Colocalizes with TCR

Microclusters in Response to Stimulation with T102L-I-E^k Preactivated AND T cells were incubated with fluid lipid bilayers containing T102L-I-E^k, CD80 and ICAM-1.
Images of LAT-EGFP and TCRβ were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 μm

**Supplementary Movie 5 (Related to Figure 5): LAT Co-localizes with TCR microclusters in Response to Stimulation with β-2m-I-Ek** Preactivated AND T cells were incubated with fluid lipid bilayers containing β-2m-I-Ek, CD80 and ICAM-1. Images of LAT-EGFP and TCRβ were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 μm

**Supplementary Movie 6 (Related to Figure 6): Grb2 Colocalizes with TCR**

**Microclusters in Response to Stimulation with MCC-I-Ek** Preactivated AND T cells were incubated with fluid lipid bilayers containing MCC-I-Ek, CD80 and ICAM-1. Images of Grb2-EGFP and TCRβ were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 μm

**Supplementary Movie 7 (Related to Figure 6): Grb2 Colocalizes with TCR microclusters in Response to Stimulation with K99A-I-Ek in the Presence of CD80**

Preactivated AND T cells were incubated with fluid lipid bilayers containing K99A-I-Ek, CD80 and ICAM-1. Images of Grb2-EGFP and TCRβ were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 μm

**Supplementary Movie 8 (Related to Figure 6): Grb2 Colocalizes with TCR microclusters in Response to Stimulation with K99A-I-Ek in the Absence of CD80**
Preactivated AND T cells were incubated with fluid lipid bilayers containing K99A-I-E\(^k\) and ICAM-1. Images of Grb2-EGFP and TCR\(\beta\) were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 \(\mu\)m

**Supplementary Movie 9 (Related to Figure 6): Grb2 Colocalizes with TCR microclusters in Response to Stimulation with T102L-I-E\(^k\)** Preactivated AND T cells were incubated with fluid lipid bilayers containing T102L-I-E\(^k\), CD80 and ICAM-1. Images of Grb2-EGFP and TCR\(\beta\) were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 \(\mu\)m

**Supplementary Movie 10 (Related to Figure 6): Grb2 Colocalizes with TCR microclusters in Response to Stimulation with \(\beta\)-2m-I-E\(^k\)** Preactivated AND T cells were incubated with fluid lipid bilayers containing \(\beta\)-2m-I-E\(^k\), CD80 and ICAM-1. Images of Grb2-EGFP and TCR\(\beta\) were simultaneously acquired every 10 seconds for 5 min. Scale bar = 5.0 \(\mu\)m