T-cell triggering thresholds are modulated by the number of antigen within individual T-cell receptor clusters

Boryana N. Manza, Bryan L. Jackson, Rebecca S. Petiti, Michael L. Dustin, and Jay Groves

Histidine-tagged variants of MHC class II, intercellular adhesion molecules-1 (ICAM-1), and CD80 for costimulation experiments are linked to the membrane through Ni2+-chelating lipid groups (14, 15). The lipids and proteins diffuse freely and as monomers on the supported membrane (16) (Fig. S1). Peptide composition and pMHC density are under direct experimental control. TCR clustering is controlled through grid patterns of metal lines, which have been prefabricated onto the underlying solid substrate. These structures, known as diffusion or mobility barriers (12, 16), block lateral transport of lipids and supported membrane associated proteins. Molecules diffuse freely within each corral, but are unable to hop between separate corrals. As the TCR engage antigen pMHC at high antigen densities, clustering ensues until all pMHC within a single corral of the grid-partitioned supported membrane are coalesced into a single cluster with their cognate TCR receptors (12) (Fig. 1D and Fig. S2).

The number of pMHC within each supported membrane corral determines the maximum pMHC content of the corresponding TCR cluster that may assemble on the T cell. Thus adjusting the grid size at constant pMHC density titrates the maximum number of pMHC per TCR cluster without changing the number of antigens engaged by the T cell. The total number of TCR and other signaling molecules within clusters is not limited by the substrate partitions. We refer to this physical manipulation of molecular organization within living cells as a spatial mutation (12, 17, 18). In the present application, T cells differing only in the peptide agonist distribution among TCR clusters are generated and compared side-by-side.

The primary goal of the present study is to determine how agonist distribution among TCR clusters governs T-cell activation. We perform a two parameter titration experiment in which the overall antigen peptide surface density as well as its partitioning among TCR clusters are independently controlled in living T cells. The results indicate that the threshold antigen densities for triggering Ca2+ flux (in terms of the number of antigens per cell) are dependent on agonist partitioning among TCR clusters. Most significantly, when antigen dose-response functions obtained on different grid partition sizes are analyzed in terms of the maximum number of antigens per TCR cluster (determined by pMHC content within individual membrane corrals), they collapse onto a single curve.

We observe that T cells trigger at an average agonist density of approximately two per TCR cluster, irrespective of the total number of agonist engaged by the cell. The term triggering threshold is used here to describe the average density at which
Fig. 1. Immunological synapse on partition patterns. (A) Schematic of experimental strategy. Primary mouse T cells interact with MHC and ICAM-1 functionalized patterned supported lipid bilayer membrane. MHC density is constant, MHC is preloaded with an appropriate ratio of null (T102E) and agonist (MCC) peptide. Chromium lines prefabricated on the glass are diffusion barriers to all lipids and lipid-tethered proteins. (B) En face view of TCR distribution in the immunological synapse. TIRF images of anti-TCR Fab on 0 MCC–MHC μM−2 1 min after contact (diffuse TCR), 1 MCC–MHC μM−2 10 min after contact (small spread TCR clusters), and 100 MCC–MHC μM−2 10 min after contact (TCR cluster). Image intensity is scaled to demonstrate clearly the TCR distribution in each case. (Scale bar: 5 μm.) (C) Percent of cells with the TCR distribution phenotypes in B. Ten minutes after contact with bilayer from a representative experiment; n = 140–150 cells per concentration. Cells with both a large central cluster and peripheral small clusters are scored in the central large cluster category, because some TCR transport to the center has occurred. (D) Bright field, reflection interference contrast microscopy (RICM), and TCR distribution of cells off grid, and on 0.5, 1, and 0.5 μm side partition grids after 5–10 min of contact with the surface. TCRs cluster until there is one TCR cluster per box; 100 ± 5% SD MCC–MHC μM−2. (Scale bar: 5 μm.)

Results

Physical Partitioning of T-Cell Receptor Clusters. T-cell activation is investigated in a hybrid immunological synapse consisting of primary mouse T cells interacting with functionalized supported lipid bilayer membranes (Fig. 1A). This configuration is widely used and has been instrumental in characterizing the immunological synapse (8, 12, 13). Glass substrates organize spontaneous self-assembly of supported membranes from small unilamellar vesicles. A characteristic feature of supported membranes, which is critical for these experiments, is the free lateral mobility of lipids and membrane associated proteins (12). For hybrid immunological synapse reconstitution, the supported membrane is functionalized with ICAM-1 and MHC loaded with various combinations of agonist [moth cytochrome c (MCC)], null (T102E), and coagonist null (ER60) peptides (8, 12, 13). For CD28 costimulation studies, CD80 is included in the supported membrane as well. The cytoplasmic domains of these proteins are expressed as histidine-tag constructs (His10 for ICAM-1 and CD80 and 2XHis6 for MHC) and stably linked to the supported membrane by multivalent interactions with Ni2+-chelating lipids mixed in the membrane. We employ kinetic control parameters to achieve desired densities of bound protein (14, 15).

AND TCR effector T cells form hybrid junctions with these supported membranes irrespective of the peptide displayed by the MHC, due to the presence of ICAM-1. When all of the MHC is loaded with the null T102E peptide, T cells spread on the surface and exhibit a relatively homogeneous distribution of TCR (Fig. 1B). At low concentrations of MCC–MHC, TCR organize into clusters throughout the contact zone. At much higher MCC–MHC, these TCR clusters are actively driven toward the center of the interface by an actin polymerization-dependent process, forming the classical immunological synapse pattern (8) (Fig. 1B and C). TCR cluster formation is coincident with and necessary for activation of downstream calcium flux, but TCR clusters can continue to form after activation (8).

Physical structures on the underlying supported membrane substrate can be used to control TCR clustering in the living cell (Fig. 1D). Barriers to lateral mobility of supported membrane components are created by metal lines (5-nm high, 100-nm wide) that are prefabricated onto the glass substrate by electron beam lithography (Figs. S1 and S3). Grid patterns of barriers trap fixed numbers of pMHC, along with any other supported membrane proteins, within each confined grid corral without otherwise interfering with their mobility. As TCR engage these mobile but trapped pMHC, they also become subject to the substrate-imposed constraint. Generally, we observe TCR to accumulate into one microcluster within a single corral. Thus the number of pMHC per corral determines the maximal number of pMHC per TCR cluster, whereas the number of TCR per cluster is not limited. TCR outnumber MHC in most clusters (Fig. S4) and especially at low pMHC densities. We note that this observation does not indicate that individual TCR-pMHC interactions are long-lived or stable (19); it indicates only that the TCR cluster is stable at high antigen density. Importantly our experiments restrict the restriction of signal strength to receptors bound only to a single antigen. In conventional experiments, TCR-pMHC interactions are often quantified in a manner that assumes receptor clustering is a direct measure of signal strength, which is inaccurate in the case of the immunological synapse. This is because the signal strength is controlled by the interaction of TCR-pMHC and not just the number of receptors involved in the interaction (16). While receptor clustering is indicative of activation, the receptor density is not (Fig. S5).

Partitioning of Agonists Among TCR Clusters Alters the Threshold Density to Trigger Intracellular Calcium Flux. The experimental ability to independently control pMHC distribution among TCR clusters enables characterization of TCR cluster signaling with unprecedented specificity. A typical experiment is depicted in Fig. 2. A population of T cells is allowed to interact with a supported membrane that is partitioned by substrate patterns in one region and unpatterned elsewhere. T-cell triggering is monitored by intracellular Ca2+ flux using fluorescence ratio imaging of fura-2 dye (13, 23) (Fig. 2A and B, and Fig. S6). Although calcium flux is a quick cellular response (24), population calcium flux is integrated over 20 min to include cells responding at different times and provide better signal to noise. The choice of integration time does not alter overall results (Fig. S7). The supported membrane is of uniform composition over the entire substrate. Thus differences in T-cell triggering on and off patterned regions result exclusively from differences in TCR-pMHC cluster size. Lymphocyte function-associated antigen 1 and ICAM-1 clustering is not altered, because each corral contains dozens of ICAM-1 mole-
In the experiments described above, agonist MCC peptide was titrated into a majority background of the null peptide, T102E. This facilitated the aggregation of homogeneous MHC, the probability of directly interpreting TCR signaling and are discussed further below. Minimal Number of Agonist Per Signaling TCR Cluster. One is not enough, but what is the minimum number of agonist pMHC per TCR cluster sufficient for triggering? We explore this question through a series of experiments in which agonist pMHC density is titrated off and on two different grid sizes, 1 and 0.5 μm. As seen in Fig. 3A, grid size and TCR-pMHC cluster size get smaller, the activation density for triggering Ca\(^{2+}\) flux becomes progressively higher. At sufficiently high concentration of MCC–MHC, cells can activate on both grid sizes. If these independent titration curves are instead plotted in terms of the number of agonist pMHC per cluster, then the 1- and 0.5-μm activation curves collapse to a single dose-response function (Fig. 3B). At average of one MCC–MHC per box, T cells do not activate, but at two they do; this result is irrespective of the total number of agonist pMHC engaged by the cell or the dimensions of the grid box. The fact that there are fourfold more TCR clusters in the more finely partitioned 0.5-μm grid cells does not affect this threshold. Additional increases in Ca\(^{2+}\) triggering as agonist pMHC density increases further are much more gradual. The onset of triggering occurs abruptly once a critical number of agonist pMHC per TCR cluster is reached. This fundamental observation has been repeated no less than nine times with populations of T cells from at least four different mice and observed with GPI-tethered proteins as well (SI Text and Figs. S8 and S9). At such low densities, the stochastic distribution of agonist pMHC among the many TCR clusters in each cell must be considered. In our experimental procedure, both agonist and null peptide loaded MHC are exposed to the membrane from solution simultaneously (26). Therefore we expect uncorrelated binding of both peptide–MHC complexes. The peptide off rate is extremely slow and peptide reloading from solution is negligible at neutral pH (27). Thus the original loading configuration is expected to persist throughout the entire experiment, which results in a Poisson distribution for the number of agonist MCC peptides in each grid box. The probability of a given TCR cluster having a particular number of agonist pMHC for different average numbers of agonist pMHC per cluster is plotted in Fig. 3C. Next, we compare the distribution of agonist among TCR clusters at an average MCC–MHC density of one per cluster, which was not activating, to an MCC–MHC density of two per cluster, which was activating, as traced by gray lines between Fig. 3B and C. Notice that the probability of two MCC–MHC in a single cluster is relatively unchanged and the probability of two or more changes by less than twofold between the nonactivating and activating situations. In contrast, the number of TCR clusters with three and, to an even greater extent, four or more MCC–MHCs has risen sharply (Fig. 3D). These results indicate that the most probable minimal triggering unit contains at least four agonist pMHC in a single TCR cluster and could require as many as six. It is unlikely that seven or more agonists are required because the probability of these becomes so low that many cells will have no TCR clusters with this many agonists.
There is another group of endogenous peptides, referred to as coagonists, that do not activate by themselves at any density but enhance activation in the presence of agonist pMHC (5, 28). A peptide from the cysteine protease of the endoplasmic reticulum (ER60) has been reported as a coagonist peptide to effector T cells, bearing the 5C.C7 TCR receptor that also recognizes MCC–MHC (5). MCC titration experiments were also performed by dilution into a background of ER60 (Fig. 4). Triggering thresholds with ER60 are the same as measured for MCC titrations into the absolute null T102E, and exhibit the same universality on a per TCR cluster basis. However, ER60 did yield an apparent increase in the magnitude of Ca\(^{2+}\) flux.

The costimulatory molecule CD80 can also modulate T-cell signaling by activating CD28 on the T-cell surface (29, 30). Inclusion of CD80 (approximately 300 molecules \(\mu m^{-2}\)) (14) in the supported membrane during MCC:T102E titration experiments yields two distinct effects on TCR signaling (Fig. 5A). First, the triggering threshold is notably shifted to lower agonist pMHC densities. Additionally, the apparent magnitude of Ca\(^{2+}\) flux was increased, in a manner similar to that observed with the coagonist ER60 peptide. This stronger activation with CD80–CD28 costimulation has been observed before (29).

Renormalization of the MCC:T102E titration responses from both grid sizes in terms of the number of antigen per grid box again yields a singular response curve (Fig. 5B). We observe that triggering thresholds are shifted by CD80–CD28 costimulation, but they are still defined on a per TCR cluster basis, rather than a per cell basis. A stochastic analysis of agonist distribution among clusters as done above reveals the most probable minimum activating unit in the presence of CD80–CD28 costimulation is two agonist pMHC per TCR cluster (Fig. 5 C and D). The data are a bit rougher at these very low triggering thresholds, but it is important to realize that if a single agonist peptide could cause a TCR cluster to trigger, then there would be no partition effect. The data presented here are not consistent with individual agonist activating a TCR cluster.

**Discussion**

The difference between the measured triggering threshold based on average and stochastic analyses deserves consideration. First, we note that triggering as defined by intracellular Ca\(^{2+}\) is essentially digital. The onset of Ca\(^{2+}\) flux is abrupt (Fig. S6E). The next question then concerns whether individual TCR clusters trigger with an all-or-nothing or a graded response. Based on the experimental evidence reported here, it is unlikely TCR clusters could yield a graded response at minimal antigen levels. We arrive at this conclusion based on the fact that partitioning TCR clusters at a constant total amount of antigen leads to an abrupt cessation of triggering. If multiple partially triggered TCR clusters were
T-cell activation with respect to titration of overall MCC–MHC surface density off and on 1- and 0.5-μm partition grids, with and without CD80. The threshold density at which T cells activate in the presence of CD80 (filled symbols) is shifted to the left for cells off grid (circle), on 1-μm grid (diamonds), and on 0.5-μm grid (squares). Lines are guides to the eye. Mean calcium flux (± SEM) of more than 80 cells per concentration, from a representative experiment. Data without CD80 are the same as Fig. 3A. Null peptide is T102E for all data points. (B) T-cell activation with respect to number of MCC–MHCs per box at 1-μm (diamonds) and 0.5-μm (squares) partition grids, in the presence of CD80. The activation profiles for 1- and 0.5-μm grids is the same in terms of number of MCC–MHC per grid box. (C) Theoretical Poisson distribution of the probability of discrete number of MCC–MHC per box at different average concentrations per box. At nonactivating densities (left gray line), most boxes have zero or one MCC–MHC; the probability goes from negligible (0.5%) to appreciable (18%) and rises sharply between non- and activating concentrations, in the presence of CD80.

Our experimental results reveal abrupt increases in the probability of triggering intracellular Ca²⁺ flux corresponding with the appearance of TCR clusters at least four agonist pMHC in the absence of costimulation.

Observation with CD80–CD28 costimulation reveal that TCR triggering thresholds are not absolute. CD80–CD28 costimulation is one of perhaps multiple ways that triggering thresholds may be tuned in vivo (5, 19, 30, 33, 34). Naïve T cells, which are dependent on this costimulation, may have much higher signaling thresholds. The one truly invariant observation among all of the results presented here is that triggering thresholds are determined on a per TCR cluster basis, and not on a per T-cell basis.

Previously, direct coupling of two TCRs, by bivalent antibody or agonist dimers, has been shown to trigger T cells (5, 35). These well known facts, along with structural evidence of binding to CD4 (36, 37), naturally lead to the speculation that a TCR dimer may be the fundamental triggering unit. However, the artificial tether between agonist alters the dynamics of agonist–TCR and TCR–TCR association, possibly stabilizing it beyond physiological conditions, and therefore cannot reveal the native threshold for triggering. The experimental design used here does not enforce or require direct dimerization between agonist pMHCs.

Estimates of T-cell sensitivity to pMHC have been made with increasing precision over the past two decades because of advancements in quantitative measurements of peptide number and characterization early T-cell responses (1, 38–41). Single agonist sensitivity has been reported by fluorescence, in a cell–cell interface where multiple ligands, protein complexes, and coagonist endogenous peptides are present (1, 2, 5). There are several factors to consider when interpreting these reports in light of the results we present here. First, our definition of triggering threshold as the average antigen number at which half-maximum response is achieved may differ from earlier interpretations. We chose this definition because it converges to an informative value in the limit of a large number of experiments. Triggering threshold cannot properly be defined as the lowest level of antigen at which any triggering was observed, because there is a nonzero probability of a cell triggering with no antigen due to stochastic fluctuations; this cannot be distinguished from causal triggering on a cell–cell basis. Secondly, supported membranes differ from live antigen presenting cell (APC) experiments because costimulatory molecule and peptide antigen distributions are directly controllable. These are intrinsically unknown in live APCs, and may alter triggering thresholds (5, 42). Although the somehow integrated within the cell to yield the equivalent of one fully triggered cluster, then such discrete changes as a function of partitioning alone are not expected.

These results are most consistent with a mechanism in which individual TCR clusters trigger, all-or-nothing, based on achieving some minimum threshold. Such a response necessitates extensive cooperativity among the TCRs within individual clusters (31). It is possible that more TCRs are triggered than the number of agonists within individual clusters, at least to the extent that triggering of an individual TCR can be defined (3). The serial triggering model (3, 32) provides one mechanism for such a phenomenon. There are also reports suggesting some degree of TCR preclustering (20), which would provide a mechanism of recruiting and possibly triggering TCR not directly engaged with antigen pMHC.

With this all-or-nothing response, the probability of a TCR cluster triggering will be a function of the number of agonist in the cluster. However, it is not necessary that a specific threshold number of agonist per cluster, above which the cluster always triggers and below which it never does, even exists. Rather, all we can expect is that the probability of a cluster triggering increases with agonist number and that this increase may be particularly abrupt around a certain critical number of agonists per cluster. Our experimental results reveal abrupt increases in the probability of triggering intracellular Ca²⁺ flux corresponding with the appearance of TCR clusters at least four agonist pMHC in the absence of costimulation.
Materials and Methods

Mice and T cells. AND CD4+ T-cell blasts were prepared by in vitro stimulation of spleen and lymph node cells from F1 cross of ANDX 810.1B mice (Jackson Laboratory) with 1–2 μM MCC peptide. Cells were maintained in IL-2 every 48 hr and used on days 5 and 7.

Patterened Supported Protoclipid Bilayers. Glass slides were patterned by e-beam lithography at University of California, Berkeley Microfabrication Lab and Molecular Foundry, Lawrence Berkeley National Laboratory. Liposomes with 97.5 mol % 1,2-dioleoyl-sn-glycero-3-phosphocholine, 2 mol % 1,2-dioleoyl-sn-glycero-3[(N-5-aminocarbamoyl)luminodiace tic succinimidy] (nickel salt) and 0.5 mol % Texas Red® 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt, prepared by sonication or extrusion, were injected over substrate in Biophyte FCS2 flow chamber. His-tagged proteins were expressed in H5 cells and purified by Ni-nitritolriacetic acid affinity (14, 22). Surface density of MCC–MHC was measured with AlexaFlour conjugated MHC peptide and calibrated with supported bilayers (43).

Fluorescence Imaging. For calcium flux, cells were loaded with 1 μM fura-2-acetoxymethyl and imaged at 340 and 380 nm with emission at 510 nm via 40x Fluor objective on Quantix 57 or CoolSnap K4 cameras. Fields of view with patterns were monitored every 7 or 15 s for 20 min. Cell motion and fluorescence intensity were tracked and analyzed semiautomatically in Imaris, Metamorph, Matlab, and Excel. TCR, actin, and CD45 were imaged via 100X N.A.1.3 objective on CoolSnapHQ camera. Total internal reflection fluorescence (TIRF) was done via 100x 1.45 NA. TIRF objective on a Cascade 512B EM CCD camera.

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