Early T cell receptor signals globally modulate ligand: receptor affinities during antigen discrimination

Rafal M. Pielak1,2, Geoff P. O’Donoghue1,3, Jenny J. Lin1,3, Katherine N. Aliferis4, Nicole C. Fay2,5, Shalini T. Low-Nam6, and Jay T. Groves1,6

1Department of Chemistry, University of California, Berkeley, CA 94720; and 2Department of Molecular and Cellular Biology, University of California, Berkeley, CA 94720

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Antigen discrimination by T cells occurs at the junction between a T cell and an antigen-presenting cell. Juxtacline binding between numerous adhesion, signaling, and costimulatory molecules defines both the topographical and lateral geometry of this cell–cell interface, within which T cell receptor (TCR) and peptide major histocompatibility complex (pMHC) interact. These physical constraints on receptor and ligand movement have significant potential to modulate their molecular binding properties. Here, we monitor individual ligand:receptor binding and unbinding events in space and time by single-molecule imaging in live primary T cells for a range of different pMHC ligands and surface densities. Direct observations of pMHC:TCR and CD80:CD28 binding events reveal that the in situ affinity of both pMHC and CD80 ligands for their respective receptors is modulated by the steady-state number of agonist pMHC:TCR interactions experienced by the cell. By resolving every single pMHC:TCR interaction it is evident that this cooperativity is accomplished by increasing the kinetic on-rate without altering the off-rate and has a component that is not spatially localized. Furthermore, positive cooperativity is observed under conditions where the T cell activation probability is low. This TCR-mediated feedback is a global effect on the intercellular junction. It is triggered by the first few individual pMHC:TCR binding events and effectively increases the efficiency of TCR scanning for antigen before the T cell is committed to activation.

Significance

Antigen discrimination by T cells is based on subtle differences in binding of the T cell receptor (TCR) for its peptide major histocompatibility complex (pMHC) ligand. While such binding characteristics are readily mapped with great precision in reconstituted biochemical systems, it is less clear how these interactions are affected in the live cell environment. Here we utilize single-molecule imaging to individually resolve all of the pMHC:TCR binding events in live T cells. The quantitative measurements reveal an active feedback mechanism that globally modulates the probability of pMHC:TCR binding throughout the cell–cell interface, without affecting the unbinding rate. The result is to increase the efficiency with which TCRs scan for antigen pMHC after the first few molecular encounters have occurred.

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1Present address: L’Oréal Tech Incubator, San Francisco, CA 94105.
2Present address: Department of Cellular and Molecular Pharmacology, University of San Francisco, San Francisco, CA 94158.
3Present address: Stanford ChEM-H, Stanford University, Stanford, CA 94305.
4Present address: Applied Molecular Transport, South San Francisco, CA 94080.
5To whom correspondence should be addressed. Email: jtgroves@lbl.gov.

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interface. Inside-out activation of lymphocyte function associated antigen-1 (LFA-1) on the T cell surface (21) and changes in the cortical actin cytoskeleton (18, 22) are plausible candidates for such feedback. Notably, positive cooperativity is only observed at pMHC densities where T cells are unlikely to be activated (e.g., as measured by NFAT nuclear translocation in these experiments). At progressively higher antigen levels, corresponding to conditions under which central supramolecular activation clusters are clearly visible (23), the observed cooperativity becomes negative.

These observations expose active feedback through the TCR signaling network that modulates pMHC:TCR and other molecular binding affinities in situ. One consequence of this feedback is to increase the efficiency with which TCRs scan for pMHC after the first few agonist pMHC:TCR molecular binding events have occurred, but before the decision to activate is reached. The sensitivity of the feedback mechanism to antigen thus even exceeds the already extreme sensitivity with which T cells activate in response to antigen. Another consequence of such a feedback system is to increase the probability of multiply rebinding the same agonist pMHC (24). Under situations where extremely few or even a single agonist pMHC is available, multiple rebinding events increase the precision with which molecular properties of the agonist, such as the corresponding pMHC:TCR $k_{on}$, can be determined. Feedback that favors multiple rebinding events will thus increase the ability of TCR to discriminate among similar pMHCs based on binding kinetics. More generally, these observations underscore how chemical properties, such as binding affinities, can be dynamically manipulated in the living cellular environment.

Results

Single-Molecule pMHC:TCR Binding in Live Cells. We probe single T cell responses to TCR triggering in hybrid junctions between live primary T cells and supported lipid membranes functionalized with pMHC, the integrin intracellular adhesion molecule-1 (ICAM-1), and the costimulatory ligand CD80 (Fig. 1A). T cell spreading and TCR triggering is monitored by total internal reflection fluorescence microscopy (TIRF) imaging of a covalently attached intercellular adhesion molecule-1 (ICAM-1) and CD80 in combination with a membrane bound representative image of a single, single T cell expressing TCR (Fig. 1B). Using this approach we tracked slow-moving, bound pMHC:TCR complexes and measured the single-molecule pMHC:TCR dwell time distributions for two different TCR mouse model systems (AND and 5c.c7) and a panel of peptide ligands of varying potencies (Fig. 1C).

The average molecular dwell times ($\tau_{m}$) range from <1 s to 68 s, correlate with observed peptide potency in these studies, and are
comparable to reported bulk solution binding measurements on isolated proteins made using surface plasmon resonance (SPR) (4, 9, 25). This assay enables measurement of in situ dwell times below the reported detection limit of SPR measurements (9).

Direct readout of the binding status of single pMHC molecules also enables measurement of the fraction of bound pMHC within each individual T cell–supported membrane junction. In these experiments, every component of the reaction quotient ([TCR]_{total}, [pMHC:TCR], and [pMHC\_free]) is measured independently to calculate a parameter we here refer to as \(\tau_{\text{off}}\), which is defined as \(\tau_{\text{off}}\) = \([\text{TCR}]_{\text{free}}\cdot[p\text{MHC}]_{\text{free}}\cdot[p\text{MHC}\cdot\text{TCR}]\). This reaction quotient essentially is the in situ dissociation constant, \(K_{D}\), within the interface (Fig. 2A and B). Although the coreceptor CD4 is not explicitly mentioned here it is present in all measurements and is expected to weakly interact with MHC to form a CD4:pMHC:TCR ternary complex (26). All observations and calculations of \(\tau_{\text{off}}\) thus intrinsically include the effects of CD4 binding. Although \(\tau_{\text{off}}\) is not strictly an equilibrium parameter, typical kinetic rates of binding and dissociation (\(k_{\text{on}}\) and \(k_{\text{off}}\)) are fast compared with the timescale of experiment. Under these conditions, the fraction of bound pMHC per T cell maintains at steady state during the course of observation (Fig. 2C and Movie S1). Population average values of \(\tau_{\text{off}}\) calculated directly from single-cell measurements are comparable to equilibrium \(K_{D}\) measurements obtained from parametric fits to bulk measurements of pMHC:TCR binding in supported membranes for all three pMHC:TCR combinations (Fig. 2B and Fig. S1B). There is, however, large variation in the individual values of \(\tau_{\text{off}}\) measured for each cell, which is not the result of measurement error or stochastic noise (Fig. 2B). We speculated that this variation is not random but rather reflects systematic modulation of pMHC:TCR binding characteristics by cellular activity.

**Systematic Variation of pMHC:TCR Binding Affinity.** The dependence of \(\tau_{\text{off}}\) on pMHC density was characterized by precision titrations ranging from very low pMHC densities (~0.05 molecules per micrometer) to very high pMHC densities (~300 molecules per micrometer). For a given pMHC density, \(\tau_{\text{off}}\) values for at least 50 cells were averaged to calculate a well-defined population average, \(\tau_{\text{off}}\) (Fig. 3A and Fig. S2A). This assay is accurate over at least four orders of magnitude of pMHC density (Fig. S1 C and D). Several distinct observations regarding the nature of ligand:receptor binding in T cell intermembrane junctions are revealed by these results.

First, pMHC:TCR binding at the lowest densities, corresponding to as few as four to five individually resolved pMHC:TCR molecular binding events per cell (Figs. S1B and S2B), exhibits distinct positive feedback. This is revealed by the systematically decreasing values of \(\tau_{\text{off}}\) observed at the lowest pMHC densities (Fig. 3A and Fig. S2A). The mean pMHC:TCR dwell time, \(\tau_{\text{off}}\), remains constant over the same pMHC density range, indicating that the pMHC:TCR \(K_{D}\) is modulated (Fig. 3A and Fig. S2B).

Second, pMHC:TCR binding exhibits positive cooperativity without physical contact between pMHC:TCR complexes. Every pMHC:TCR molecular complex is directly resolved and is observed to be spaced microns apart at the lowest pMHC densities tested (Figs. 1B, 2A, and 3D). This is revealed by the systematically decreasing values of \(\tau_{\text{off}}\) observed at the lowest pMHC densities (Fig. 3A and Fig. S2A). Thus, under conditions at which TCR microclusters are readily observed (22, 23, 27) (Fig. S2C), pMHC:TCR binding is antioperative—contrary to common assumption.

The pMHC densities at which maximum pMHC:TCR affinity is measured coincide with T cell activation thresholds for all three pMHC:TCR combinations (Fig. 3B and Fig. S2A). NFAT translocates to the nucleus in response to sustained calcium...
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PMHC:TCR binding affinity

Modulation of pMHC:TCR affinity occurs through changes in \( k_{\text{on}} \) of the pMHC:TCR interaction. \( k_{\text{on}} \) is a contextual parameter that is intrinsically affected by the intermembrane environment (29–31). We therefore hypothesized that the first few pMHC:TCR binding interactions could trigger changes in the T cell:APC interface, which in turn increased \( k_{\text{on}} \) of subsequent pMHC:TCR binding. Such a general morphological mechanism should also produce similar effects on other juxtacrine ligand:receptor interactions at the interface. Both CD80:CD28 and pMHC:TCR complexes have intermembrane distances of \( \sim 13 \) nm and bind with comparable solution affinities in the low micromolar range (25, 32–34) (Fig. 4.4). Therefore, if a general change in membrane morphology is enhancing pMHC:TCR binding, this effect should also be experienced by other, similarly sized intermembrane ligand:receptor complexes, such as CD80:CD28.

We varied the unlabeled pMHC density and monitored single-molecule binding kinetics of the CD80:CD28 costimulatory interaction using the same imaging strategy applied to pMHC:TCR (Fig. 4.4). Observations of individual CD80:CD28 binding events reveal a similar increase in binding efficiency at the same pMHC densities that maximized pMHC:TCR affinity for both MCC and T102S peptides (Fig. 4B and Fig. S3 A and B). Histograms for each condition are populated from AND CD80+ T cell clones and three separate mice. Well-resolved CD80:CD28 binding events are spaced microns apart (Fig. 4C and Fig. S3C) and their intensity distribution remains constant when pMHC density is varied (Fig. S3C), which demonstrates that the increase in CD80 affinity is not due to enhanced CD80:CD28 dimerization (35). Notably, this cross-talk effect of pMHC:TCR binding on CD80:CD28 affinity is not reciprocal. Addition of (unlabeled) CD80 does not appreciably shift pMHC:TCR \((K_D)\) [the difference in \((K_D)\) minima with and without CD80 \((0.15)\) is within the SE in the pMHC titration measurement \((0.11–0.15)\)], indicating that CD80:CD28 binding does not contribute to the cooperative effect (Fig. 4D). Addition of CD80 lowers the NFAT translocation threshold density for the agonist MCC ligand (36), but this effect is less prominent for the weaker T102S ligand (Fig. 4E and Fig. S3D). CD80:CD28 complexes travel along linear trajectories, confirming effective engagement with the T cell cytoskeleton (Fig. S3E).

Similar to pMHC:TCR, the CD80:CD28 \( (\tau_{\text{off}}) \) is constant as a function of pMHC density (Fig. S3F).

These results reveal that positive feedback generated by early pMHC:TCR binding events also increases \( k_{\text{on}} \) for CD80:CD28, while CD80:CD28 binding exhibits no reciprocal effect on pMHC:TCR. Since CD80:CD28 and pMHC:TCR complexes are physically similar, and are therefore expected to exert similar mechanical perturbations on the intermembrane environment, the lack of reciprocal binding cooperativity suggests a passive physical mechanism of membrane pinning is not responsible for the observed cooperativity. Instead, the mechanism appears to involve morphological changes in the interface triggered by signaling activity of pMHC:TCR.

**Cytoskeleton and Integrin Signaling Contribute to pMHC:TCR Affinity Enhancement.** Both pMHC:TCR and CD80:CD28 complexes clearly engage the cytoskeleton at densities below NFAT threshold where affinity enhancement is observed. Additionally, integrins play an important role in establishing the physical geometry of the intercellular interface. Both effects could modulate ligand:receptor binding, and we examine these possibilities here with inhibitor studies. We probe the role of cytoskeleton activity in pMHC:TCR affinity enhancement using the small-molecule inhibitor Latrunculin A (LatA), which disrupts actin polymerization by binding G-actin. Effects of integrin signaling were probed using GGT1-298, a geranylgeranytransferase I

release, and confocal microscopy imaging of NFAT subcellular localization provides a visual, binary readout of individual T cell activation (Fig. 3C). For each peptide, the activation threshold is the density at which half of the maximum fraction of cells translocate NFAT after 30 min of pMHC stimulation (28) (Fig. 3B and Fig. S2 A and B). At pMHC densities below the activation threshold, where T cells are scanning for and engaging antigen but have a low probability of activation (NFAT translocation), we measure positive cooperativity in pMHC:TCR binding. At higher pMHC densities, where T cells are likely to be activating, we measure negative cooperative binding between pMHC and TCR (Fig. 3 A and B and Fig. S2). Finally, the feedback strength, which is reflected in the slope of change in \((K_D)\) with pMHC density, depends on \( (\tau_{\text{off}}) \) of the stimulating ligand (Fig. S2 A and B). Thus, there is kinetic discrimination in this effect, which suggests TCR triggering is involved.

Here, we refer to these TCR-mediated effects that are initiated by as few as five pMHC:TCR binding events and are insufficient to induce NFAT activation as “early TCR signals” (Fig. S2). This TCR-mediated feedback depends on pMHC:TCR strength (Fig. S2B), but the precise signaling pathways remain to be mapped.
Dose titrations of each inhibitor were performed at pMHC densities close to and below the maximum affinity for the MCC/MHC:AND interaction (Fig. 5A). Titrating the dose of GGTI-298 resulted in a monotonic decrease in MCC:TCR affinity enhancement, and titrating the dose of LatA leads to a similar, but smaller, decrease in affinity enhancement. At the optimum density, phallolidin staining after T cell fixation revealed the expected enrichment of F-actin at the T cell periphery in the absence of LatA and a relatively even distribution of F-actin in the presence of LatA (Fig. S4A). Inclusion of a recombiant ICAM-YFP fusion in the supported membrane revealed the expected ring-like ICAM distribution at the periphery of control T cells (Fig. S4B). Rap1 inhibition not only disrupted this distribution of ICAM, resulting in a relatively even ICAM-YFP distribution across the T cell, but also decreased the probability of the T cell's landing on the supported membrane. These cytoskeletal and adhesion effects may be related to density- and \( \tau_{\text{off}} \)-dependent trends in T cell landing on pMHC-conjugated supported membranes (Fig. S4C). Inhibition of either actin polymerization or Rap1 activity alters the pMHC:TCR affinity modulation and thus indicates a mechanistic role for both cytoskeleton and integrin inside-out signaling in pMHC:TCR affinity enhancement.

**TCR-Mediated Morphology, Adhesion Dynamics, and Proximal Signaling.** The decreasing trend in \( K_D \) before NFAT activation indicates that T cells become more responsive to their surroundings after a few initial binding events. This enhanced sensitivity is also reflected in changes in cell morphology, adhesion, and proximal signaling at pMHC densities where NFAT activation is not observed. At T102S/MHC densities well below NFAT threshold, T cells adopt an asymmetric, crawling morphology and exhibit low levels of Zeta-chain-associated protein kinase 70 (ZAP70) recruitment to the plasma membrane (Fig. 5B). Identical T cells exposed to MCC/MHC densities above NFAT translocation threshold exhibit a stationary, centro-symmetric cell morphology and enhanced ZAP70 recruitment. T cell crawling leads to binding of fresh pMHC and correlated ZAP70-EGFP recruitment in the newly engaged region of the supported membrane (Fig. 5C). The transition between these modes of behavior is mediated by TCR triggering and correlates with \( K_D \) changes reported here.

**Discussion and Conclusion**

Even at pMHC densities well below NFAT translocation thresholds we observe T cells to exhibit a global response to antigen that modulates the binding affinities of ligand:receptor complexes within the interface. We have previously reported that individual pMHC:TCR binding events have been observed to elicit macroscopic changes in cytoskeleton behavior (12). This effect, also readily observed in the data presented here (Fig. 1B and Fig. 5), illustrates how different signaling pathways triggered by the TCR can have different set points for activation. We find that, depending on the pMHC:TCR interaction and the composition of the T cell clone, at the lowest densities measured (corresponding to as few as approximately five steady-state pMHC molecular binding events per cell), T cells can activate retrograde transport of pMHC:TCR complexes and induce an increase in affinity for pMHC. Although the effects we report here are not spatially localized, this positive feedback may be enhanced by mechanisms dependent on close physical proximity of pMHC:TCR complexes (38) and by interplay between local Ca\(^{2+}\) release and actin polymerization at the supported membrane: T cell interface (39). By contrast, at densities that result in half-maximal NFAT nuclear translocation in both AND and 5c.7 systems, T cells have at least 30 simultaneous pMHC:TCR engagement events per cell (Fig. S2F). Addition of the costimulatory molecule CD80 shifts NFAT triggering thresholds to lower pMHC densities (Fig. 4E).

The ability of T cells to respond to a small number of pMHC (here observed by modulating \( K_D \)) without fully activating may inhibitor that targets GTPase Rap1 (37). Rap1 activates inside-out signaling via interactions with LFA-1 (21).

![Fig. 5. Cytoskeleton and integrin signaling contribute to pMHC:TCR affinity enhancement. (A) The fold change in the fraction of bound MCC-Atto488/MHC at the optimum MCC density relative to the lowest MCC density decreases in the presence of Rap1 and actin inhibition for AND T cells. n ≥ 15 for each condition. Error bars indicate SEM. (B) ZAP70-EGFP speckles in AND T cells are imaged using TIRF microscopy. Each blue dot represents a ZAP70-EGFP speckle; the positions of every ZAP70-EGFP feature collected over an ∼5-min window are projected onto a single image for each cell. Linear ZAP70-EGFP trajectories are visible in T cells exposed to either MCC/MHC or T1025/MHC. These trajectories are centrosymmetric in the case of MCC/MHC and asymmetric in the case of T1025/MHC. (C, Left) An AND T cell scavenges for MCC/MHC over an ∼120-s period. An edge detection algorithm using ZAP70-EGFP in TIRF as a contrast agent detects the cell outline. Individual MCC-Atto647N/MHC molecules (blue dots) are tracked in TIRF. (C, Top Right) Cell movement corresponds with an increase in pMHC binding. *, **, and *** denote time points as indicated in the images at Left. (C, Bottom Right) The pMHC molecule indicated by the red dot at \( t = 94 \) s reveals localized recruitment and correlated movement of ZAP70-EGFP at the site of pMHC:TCR binding. (Scale bar, 5 μm.)](image-url)
provide a mechanism by which individual T cells reduce their error rate during antigen detection. This would enable T cells to transiently slow down and check for more antigen on a particular APC, or to rebind the same antigen for multiple measurements of its dwell time. The cell may keep crawling if the initial binding events do not lead to sufficient additional pMHC:TCR binding to peptide/I-Ek complexes: Correlation of the dissociation rate with T-cell responsiveness. Proc Natl Acad Sci USA 91:12862–12866.

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