Published in final edited form as:

J Immunol. 2014 July 1; 193(1): 68–76. doi:10.4049/jimmunol.1303436.

Accumulation of serial forces on TCR and CD8 frequently applied by agonist pMHC triggers calcium in T cell

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Abstract

T cell activation by antigen is one of the key events in adaptive immunity. It is triggered by interactions of the T-cell receptor (TCR) and coreceptor (CD8 or CD4) with antigenic peptides embedded in the major histocompatibility complex (pMHC) molecules expressed on antigen presenting cells (APCs). The mechanism of how signal is initiated remains unclear. Here we complement our two-dimensional (2D) kinetic analysis of TCR–pMHC–CD8 interaction with concurrent calcium imaging to examine how ligand engagement of TCR with and without the coengagement of CD8 initiates signaling. We found that accumulation of frequently applied forces on the TCR via agonist pMHC triggered calcium, which was further enhanced by CD8 cooperative binding. Prolonging the intermission between sequential force application impaired calcium signals. Our data support a model where rapid accumulation of serial forces on TCR–pMHC–CD8 bonds triggers calcium in T cells.

Introduction

T-cell effector functions result from activation of signaling cascades triggered by interactions of the T-cell receptor (TCR) with antigen peptides presented by major histocompatibility complex (pMHC)(1, 2). TCR–pMHC interaction in cooperation with coreceptor CD4 or CD8 initiates phosphorylation of the immunoreceptor tyrosine-based activation motifs of the CD3 by the lymphocyte-specific protein tyrosine kinase p56^{lck}, which allows docking of the zeta-chain-associated protein kinase 70. Activation signals are transduced by the coordinated phosphorylation of additional protein kinases, recruitment of the adaptor molecules such as linker for activation of T cells, and activation of the

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Disclosures: The authors have no financial conflicts of interest.

phospholipase C γ (1). This initiates the production of diacylglycerol and inositol-1,4,5-trisphosphate, which rises cytosolic calcium by Ca²⁺ release from intracellular stores and Ca²⁺ entry from activated store-operated channels in the plasma membrane (3-5). The level and duration of the Ca²⁺ flux together with other signaling events determine the downstream T-cell response (6).

Since TCR is the only molecule on the T-cell surface that interacts with specific antigen, the kinetic parameters of its interaction with pMHC provide the first-level control of downstream T-cell effector functions (7, 8). However, it is unclear how pMHC binding to the membrane-distal end of the TCR causes the defined biochemical changes in the cytoplasmic domains of the associated CD3 that initiate the signaling cascade. Several models have been proposed to explain how the signal embedded in the TCR–pMHC binding is transduced across the T-cell membrane. Proposed mechanisms include TCR mechanosensor (9), receptor deformation/conformational changes (9-14), kinetic segregation (15), TCR clustering (16), permissive geometry (17), signaling chain homooligomerization (18), and serial engagement (7, 19). Yet, the detailed mechanisms remain unresolved.

Previously, we used the *micropipette adhesion frequency assay* (20, 21) to analyze *in situ* kinetics of TCR–pMHC (7, 22, 23) and pMHC–CD8 (22, 24) bimolecular interactions as well as TCR–pMHC–CD8 (22, 25) trimolecular interaction on the surface of living T cells. These two-dimensional (2D) measurements are found to correlate with T-cell activation better than their three-dimensional (3D) counterparts measured by surface plasmon resonance (SPR) (7, 22, 23, 26). However, the functions examined in cytokine secretion and proliferation studies are quite distant from the initial TCR triggering event, occur in time scales far longer than pMHC binding of TCR and/or coreceptor, and require separate assays performed under different conditions from that of the 2D kinetic measurement. To address this shortcoming, we combined the high temporal resolution micropipette 2D kinetic analysis with concurrent calcium imaging in this study, since calcium mobilization occurs rapidly after TCR engagement (27-29). We found that calcium was triggered by accumulation of frequently applied serial forces on TCR and/or CD8 via agonist pMHC.

Materials and Methods

Cells and proteins

Naive CD8⁺ T cells from OT1 transgenic mice were obtained using an Emory University IACUC-approved protocol. The following peptides were synthesized: ovalbumin-derived peptides OVA (SIINFEKL), A2 (SAINFEKL), G4 (SIIGFEKL), E1 (EIINFEKL) and R4 (SIIRFEKL), and a vesicular stomatitis virus-derived peptide VSV (RGYVYQGL) (7). OVA, A2, E1, and R4 are recognized by the OT1 TCR but VSV is a null peptide. Monomeric mouse pMHC-I H-2K^b with C-terminal biotin tags and pMHC-I mutant (OVA:H-2K^b α 3A2) were produced by the NIH Tetramer Core Facility. PE-conjugated anti-mouse TCR V α 2 (B20.1) and V β 11 (RR3-15) were from BD Biosciences (San Jose, CA). Anti-mouse H-2K^b (3H2672, PE-conjugated) was from US Biological (Swampscott, MA) and Biocarta (San Diego, CA), respectively. Anti-biotin (Bio3-18E7.2, PE-conjugated) was from Miltenyi Biotec (Auburn, CA). Intracellular calcium indicator Fura-2/AM was from Invitrogen (San Diego, CA).

Coating pMHC onto RBCs and determining site densities

RBCs were isolated with a Georgia Institute of Technology IRB-approved protocol as described (7). To coat different pMHC densities, RBCs were biotinylated with Biotin-X-NHS (EMD) at different concentrations as described (24). Biotinylated RBCs were incubated with excess streptavidin for 30 min and with saturating amount of pMHC for another 30 min after removing unbound streptavidin. The densities of pMHC, TCR, and CD8 were determined by flow cytometry as described (7).

Determining RBC membrane tension

RBC membrane tension (T_{RBC}) was determined from the suction pressure (P) and the respective radii of the micropipette (R_p) and the spherical portion of the RBC outside the pipette (R_{RBC}) using the Law of Laplace, which results in the following formula (30).

$$T_{\text{RBC}} = \frac{\Delta P}{2} / \left(\frac{1}{R_p} - \frac{1}{R_{\text{RBC}}} \right) \quad (1)$$

Dual micropipette system for 2D kinetic analysis

We customized an inverted microscope (TMD Diaphot, Nikon) by mounting two identical sets of 3D mechanical manipulators (Newport) on each side of the microscope stage to hold opposing glass pipettes for capturing a pair of cells. A computer with a LabVIEW software (National Instruments) controlled the one-dimensional open-loop piezo actuator LVPZT (P840-1, Physik Instrumente) mounted on one side of the manipulator to drive its approach and retract movements. Using an analog CCD camera (MTI DC330, Dage), we acquired real-time images at 30 frames per second and simultaneously observed them through a TV monitor and recorded by a VCR.

The micropipette adhesion frequency assay has been described (20, 21). Briefly, a T cell and a RBC were aspirated by respective pipettes (Fig. 1) and driven to contact with controlled area A_c and duration t_c . Adhesion was observed from stretching of the RBC on T cell retraction. Each T-cell–RBC pair was tested for a total of 10 min during which the contact-retract cycle at a given contact duration was repeated. 10–12 cell pairs were used to estimate an adhesion frequency P_a (mean $\pm$ s.e.m.) at each given contact duration, which was calculated as the number of adhesions N_a divided by the number of total contacts N_c .

Calculations of the number of bonds and their lifetimes accumulated over the 10-min experimental period

The average number of bonds $\langle n \rangle$ present at any time during a contact can be calculated from the adhesion frequency P_a (7, 20):

$$\langle n \rangle = -\ln(1 - P_a) \quad (2)$$

Due to the rapid 2D kinetics of the OT1 TCR–OVA:H-2K^b α 3A2 interaction (Fig. 4A) (7), at contact durations $t_c > 0.1$ s, $\langle n \rangle$ reached steady-state and is equal to:

$$\langle n \rangle = m_T m_I A_c K_a \quad (3)$$

where m_T and m_I are the respective site densities of the TCR and pMHC, and the product $A_c K_a$ of A_c (contact area) and K_a (2D affinity) is the effective 2D affinity (7, 20). Eq. 3 is obtained by setting $t_c \rightarrow \infty$ in Eq. 2 of the Online Methods of *Ref.* (7). Since k_{off} ranges from 1-10 s⁻¹ (7), a few seconds of contact time t_c is long enough to be considered as infinity because $\exp(-k_{\text{off}} t_c)$ would be negligible compared to 1. It follows from Eq. 3 that multiplying $\langle n \rangle$ by the previously measured 2D off-rate k_{off} (~ 10 s⁻¹) yields the cellular on-rate, or the frequency of bond formation during T-cell–RBC contact:

$$m_T m_I A_c k_{\text{on}} = m_T m_I A_c K_a \times k_{\text{off}} = \langle n \rangle \times k_{\text{off}} \quad (4)$$

Multiplying the frequency of bond formation (Eq. 4) by a given contact duration t_c (or the cumulative contact duration Σt_c for the entire 10-min experiment) yields the number of bonds formed at that contact N_b (or the cumulative number of bonds formed ΣN_b).

$$N_b = \langle n \rangle \times k_{\text{off}} \times t_c \quad (5a)$$

$$\sum N_b = \langle n \rangle \times k_{\text{off}} \times \sum t_c = \sum (\langle n \rangle \times k_{\text{off}} \times t_c) \quad (5b)$$

Since the reciprocal off-rate is the average bond lifetime (31), we can multiply N_b (or ΣN_b) by $1/k_{\text{off}}$ to obtain the length of time t_b during the contact when a TCR or TCRs are engaged with pMHC (or the cumulative bond lifetime Σt_b):

$$t_b = N_b / k_{\text{off}} = \langle n \rangle \times t_c \quad (6a)$$

$$\sum t_b = \sum N_b / k_{\text{off}} = \langle n \rangle \times \sum t_c \quad (6b)$$

Thus, $\langle n \rangle$ is also the fraction of contact time during which the TCR is engaged with pMHC. The calculations for the case of TCR–OVA:H-2K^b–CD8 trimolecular interaction are assumed similar.

Calcium measurements

Naïve CD8⁺ T cells were loaded with Fura-2/AM (3 μM in DMSO), incubated for 30 min at 37°C, washed twice with PBS buffer (pH 7.4). T cells and RBC pre-coated with pMHC were transferred into microscopic chamber with L-15/HEPES media (Sigma). Similar to the previously used adhesion frequency assay for 2D kinetics analysis, each T-cell–RBC pair was tested repeatedly at a given contact duration, but for a total 10 min of experimental period instead of 100 contact-retract cycles. Concurrently, dual excitation filters (340 and 380 nm) were switched automatically using a filter wheel (Sutter) to filter xenon light (Lambda LS, Sutter) to excite the Fura-2. The image acquisition and analysis was performed with using NIS-Element software (Nikon). For chelating extracellular Ca²⁺, EGTA (final

concentration 1 mM) were injected directly into chamber medium. Experiments were done at 37°C unless otherwise stated.

Statistical analysis

The statistical significance of differences were calculated using F-test and ANOVA. A p-value <0.05 is considered statistically significant.

Results

We upgraded our micropipette system by adding an optical path to enable concurrent micromanipulation and calcium imaging (Fig. S1). The micropipette adhesion frequency assay (20) uses a red blood cell (RBC) as a surrogate APC for presentation of H-2K^b MHC molecules loaded with OVA or variant peptides for TCR and/or CD8 binding. It also functions as an adhesion sensor to detect binding to a naïve OT1 CD8⁺ T cell (Fig. 1A, left panels). The presence of a bond or bonds at the end of a given contact duration t_c is registered by RBC elongation upon its retraction (lower left panel of Fig. 1A), which pulls on the TCR and/or CD8 via engaged pMHC(s) until bond rupture, allowing the approach/retraction cycle to be repeated. The adhesion frequency P_a is calculated as the number of adhesions N_a divided by the total number of test cycles N_c for a given cell pair with the same contact duration and area (Fig. 1A, right panel). Concurrently, Fura-2 ratiometric fluorescence imaging (Fig. 1B) of the cytosolic calcium concentration (Fig. 1C) in the same T cell was performed over the same 10-min period.

Serial adhesions to agonist pMHC-bearing RBC induced calcium in T cell

Repeated formation and detachment of T-cell adhesions to RBC coated with OVA:H-2K^b induced a specific increase in intracellular calcium that reached a maximum level within 4-5 min at 37°C (Fig. 1B middle row and green curves in C). Neither binding (Fig. 1A right panel) nor calcium increase (Fig. 1B top row and Fig. 1C dark blue dotted curve) was observed when the null peptide VSV:H-2K^b instead of OVA:H-2K^b was coated on biotinylated RBC or when no pMHC was coated (Fig. 1B bottom row and Fig. 1C light blue dashed curve). Cyclic T-cell contacts with surrogate APC were required as stopping the test cycles returned the Fura-2 fluorescence ratio back to the baseline level (Fig. 1D). Further, subsequent resumption of T-cell–RBC contact cycles resumed the increase in calcium (Fig. 1E). Interestingly, holding T-cell and RBC in uninterrupted contact for 10 min did not result in appreciable calcium despite that bond formation and dissociation continuously occurred at the zero-force condition (20), suggesting a requirement of mechanical pulling on the TCR and/or CD8 to trigger calcium in this time window (Fig. 1F).

The major features of the calcium time courses, shown as example in Fig. 1C, could be captured by two parameters: the maximal calcium level normalized by the initial value (I_{max}) and the calcium level measured as integrated calcium or Area Under the Curve (AUC) traditionally used to present intracellular calcium data. The strong linear correlation between these two parameters (Fig. 2A) allows us to use either parameter as a reduced representation of the calcium data in the subsequent analysis.

The Ca^{2+} response was faster and reached a higher level when measured at 37 than 25 °C (Fig. 1C). The calcium level measured as AUC at 37°C was significantly reduced by removal of extracellular calcium (by adding EGTA to chamber solution) to a level comparable to the 25°C level. Calcium level at 25°C was not affected by depletion of extracellular calcium (Fig. 2B). These results are consistent with previous reports (4, 32), confirming that the cytosolic calcium in T cells induced by pMHC engagement came from two sources. The release from intracellular calcium storage gave rise to the calcium flux that occurred at both 25 and 37°C. The additional calcium increase at 37°C resulted from calcium entry through plasma membrane channels.

Stiffening the APC increases calcium responses in T cells

It has recently been shown that T-cell signaling is enhanced by the increasing stiffness of the substrate to which the pMHC ligand is linked (33). The substrate in our experiment is the RBC adhesion sensor whose rigidity could be easily manipulated by tuning its membrane tension through changes in the micropipette suction pressure (see Eq. 1, Materials and Methods) (31). Consistent with the previous report (33), we observed that increasing the RBC membrane tension (increased suction pressure) induced higher calcium levels (Fig. 3). To achieve the piconewton sensitivity of the force transducer with a soft RBC yet trigger good levels of calcium, we used mid-range membrane tensions for all experiments in this paper except those indicated in Fig. 3. The data in Fig. 3 support the view that the TCR can sense mechanical cues presented via pMHC. For a given approach/retraction speed of the T-cell–RBC adhesion test cycle, increases in RBC membrane tension led to greater loading rates of the pulling force applied to the TCR–pMHC and TCR–pMHC–CD8 bonds, which could provide a potential mechanism for mechanosensing.

This potential mechanism may relate to a recent finding that increasing the loading rate of applied force changes the dissociation characteristics of L-selectin bond with P-selectin glycoprotein ligand 1 from a catch-slip bond with prolonged lifetime at optimal force to a slip-only bond with much shorter lifetime under higher force (34). Catch bond, a counterintuitive behavior where force strengthens the molecular interaction as opposed to slip bond that weakens it, has been suggested to provide a potential mechanosensing mechanism (9, 35, 36). We found that increasing approach/retraction speed by shortening the approach/retraction segments of the contact cycle triggered lower level of calcium in T cells (Fig. S2). These data suggest that the duration of force applied by engaged pMHC on TCR and/or CD8 may be important for their triggering. In all other figures the RBCs were driven to and from the T cells with 1-s duration each (cf. Fig. 4A).

Frequently applied intermittent forces triggers strong calcium

In addition to the approach and retraction speed, we also varied the intermission length between consecutive contacts (Fig. 4A). To do so without changing the approach/retraction speed of the RBC, we increased the pause period t_p (from the moment when the previous retraction ends to the moment when the next approach starts) from 0 to 5 and 10 s while keeping the contact duration constant at $t_c = 2$ s. Two agonist peptides presented by H-2K^b, OVA and A2, were tested using these repeated cycles for a range of constant pause periods. The pMHC densities on the RBCs were adjusted to ensure similar adhesion frequencies

(~60%) for both peptides. As shown in Fig. 4B, increasing the pause period t_p dose-dependently suppressed the calcium response to OVA engagement at experimental time > 250 s. Calcium response to A2 stimulation was similarly reduced by prolonging the pause period t_p , in particular at longer experimental time (Fig. 4C). These data indicate that reducing the frequency of force application to the TCR and/or CD8 lowers the ability to elicit T-cell calcium response.

Characterization of calcium response to cyclic adhesion tests

To correlate calcium signaling with adhesion kinetics, we contacted T cells with repeated cycles of a range of constant durations and observed the two-stage adhesion kinetics previously reported (22, 25) (Fig. 5A). The first-stage is identified by the rapid plateau in adhesion frequency P_a vs. contact duration t_c and was mediated by TCR–pMHC bimolecular interaction as shown using the mutant MHC (H-2K^bα3A2) that substituted the mouse H-2K^b α3 domain by that of human HLA-A2 to eliminate the mouse CD8 binding site. After the contact duration reached 1-s, the adhesion frequency sharply increased to a second plateau, which was CD8-dependent, as it was not observed using H-2K^bα3A2. We previously demonstrated that the second-stage adhesion is Src kinase-dependent and mediated by TCR–pMHC–CD8 trimolecular interaction (22, 25). Note that, at 50-60% adhesion frequencies, many of the adhesions could be mediated by multiple bonds. The characterization of the force applied to single bond is the subject of another publication (Liu et al., unpublished data). Using the mutant MHC to prevent CD8 binding abolished calcium response at 25°C while low calcium levels were observed at 37°C, which was insensitive to the contact duration beyond 0.1 s (Fig. 5B). The calcium signal in response to wild-type MHC was indistinguishable to that obtained using the mutant MHC at contact durations $t_c < 1$ s, which was likely due to the absence of CD8-dependent binding at short contact durations. Much higher calcium was induced by longer contact durations when adhesions were enhanced by the TCR–pMHC–CD8 trimolecular interactions. Overall calcium levels rapidly increased at 1 s and reached a maximum at 2 s at both 37 and 25°C, although levels were much higher at 37°C (Fig. 5B). These data build on the published results that the coreceptor CD8 augments the sensitivity of antigen recognition by a T cell (37). As contact time t_c increased beyond 2 s (Fig. 5B), the calcium signal decreased, suggesting that contact duration per se is insufficient to trigger calcium. This observation is also consistent with the inability of continuous contact to trigger calcium (Fig. 1F). In addition to OVA:H-2K^b, we analyzed the calcium response in T cells repeatedly contacted by RBCs bearing H-2K^b bound with several well-characterized altered peptide ligand variants, including A2 (agonist), E1 (weak agonist/antagonist), and R4 (antagonist) (7). Densities of different pMHCs on RBCs were adjusted to achieve similar adhesion frequencies (Fig. S3A-C). The variant agonist A2 induced a similar AUC vs. t_c curve (Fig. S3D) to the wild-type agonist OVA (Fig. 5B). In contrast, the lower affinity peptides (E1 and R4) failed to generate appreciable calcium at 25°C (Fig. S3E and F), consistent with the lower potencies of these weaker ligands to trigger T-cell signaling.

Accumulation of frequently applied forces on TCR and/or CD8 via engaged antigen pMHC triggers Ca^{2+} signaling

The concurrent *in situ* kinetic measurements of TCR–pMHC and/or TCR–pMHC–CD8 interactions and imaging of intracellular calcium generated two 10-min time courses for each T cell tested (cf. Fig. 1A right panel upper curve and Fig. 1C green curve). To decipher the relationships between them, we performed single-cell correlative analysis of the two time courses. We chose AUC and the maximal Fura-2 ratio I_{max} as the metrics for analyzing the calcium time course. To reduce data representation of the repeated contact cycles, we calculated the adhesion frequency P_a , the cumulative number of bonds ΣN_b predicted to form in all contacts (cf. Eq. 5b), the accumulation of bond lifetime Σt_b during all contacts (cf. Eq. 6b) when a TCR or TCRs are predicted to engage with pMHC with or without the co-engagement of CD8, and the number of observed adhesions N_a . Note that ΣN_b and Σt_b represent, respectively, the total number of bonds formed and the accumulation of their lifetimes during all cyclic contacts Σt_c in the absence of tensile force (20). By comparison, adhesions were observed only at the end of t_c when a bond or bonds (if any) were pulled by force to stretch the RBC. So N_a represents a small fraction of the total bonds formed that were pulled by force (cf. Fig. 1A).

The correlative analysis was first done by comparing the ΣN_b (Fig. 5C, left ordinate) vs. t_c , Σt_b (Fig. 5C, right ordinate) vs. t_c , and N_a vs. t_c (Fig. 5D) curves with the AUC vs. t_c curve (Fig. 5B). The cumulative number of bonds ΣN_b and their cumulative lifetimes Σt_b increased monotonically with the contact duration t_c (Fig. 5C), as expected from their respective definitions (cf. Eqs. 5b and 6b). The number of observed adhesions N_a for OVA:H2-K^b increased with t_c when $t_c < 1$ s but decreased after $t_c > 2$ s (Fig. 5D). This is because P_a increased with t_c before the second-stage adhesion (see Fig. 5A), but the total number of contact cycles repeated in the 10-min experiment, $N_c = N_a/P_a$, was reduced by increasing contact duration per cycle beyond $t_c > 2$ s. It is very telling that the AUC vs. t_c curves (Fig. 5B) do not resemble the P_a (Fig. 5A), ΣN_b and Σt_b (Fig. 5C) vs. t_c curves, but resemble the N_a vs. t_c curves (Fig. 5D). Using I_{max} instead of AUC as calcium induction metric (Fig. S4), which resembles Fig. 5B, led to the same conclusion. These results suggest that the predictor for calcium induction is not how likely adhesion occurs, how many bonds are formed, or how long these bond lifetimes accumulate during repeated T-cell–RBC contacts, but is the accumulation of these bonds that are pulled at the end of the serial contacts.

To obtain further evidence, we next plotted directly the two metrics for calcium induction vs. P_a , ΣN_b , Σt_b , and N_a and examined the correlation of (or the lack thereof) these parameters (Fig. 6). Consistent with the resemblance between Fig. 5B and Fig. 5D, and the lack of resemblance between Fig. 5B and Fig. 5A&C, repeat contacts by OVA:H2-K^b induced intracellular calcium fluxes in OT1 T cells that first increased with increasing P_a , ΣN_b and Σt_b (Fig. 6A, B, D and E). After reaching a maximum, Ca^{2+} rapidly decreased to the level comparable to that of OVA:H2-K^bα3A2, which remained very low. This calcium decrease in response to increasing P_a , ΣN_b and Σt_b refuted the adhesion frequency, cumulative number of bonds and their cumulative lifetimes as determining parameters of calcium signals. By comparison, Ca^{2+} increased with N_a as two line segments of similar

positive slopes but different levels (Fig. 6C&F). The lower segment represents calcium induction by pulling on mostly TCR–pMHC bimolecular bonds, as it corresponds to short contact durations $t_c \approx 1$ s (indicated) where the adhesion frequency curve is in the first plateau (Fig. 5A). The upper segment represents calcium induction by pulling on TCR–pMHC–CD8 trimolecular bonds, for it corresponds to long contact durations $t_c \approx 2$ s where the adhesion frequency curve is in the second plateau (Fig. 5A). Contacts at threshold duration $t_c = 1$ s produced both bi- and tri-molecular bonds, therefore inducing an intermediate level of calcium that connected the two segments. The reverse relationship between N_a and t_c is evident from Fig. 6C&F, explaining the decrease in calcium with increasing t_c as it exceeded 2 s (Figs. 5B and S3). It also explains the calcium decrease in Fig. 6B&E when $\Sigma N_b > 3500$ ($\Sigma t_b > 350$ s), because these correspond to increasing t_c and decreasing N_a . Together, these data support the hypothesis that T-cell signaling was induced by accumulation of applied pulling forces on TCR–pMHC–CD8 trimolecular bonds.

Discussion

In this paper we studied calcium responses to pMHC engagement with TCR with and without the co-engagement of CD8 in naive T cells. We found that calcium signals increased with the biological activity of the peptide (Figs. 4 and 2A) and were enhanced by cooperative binding between TCR and CD8 for pMHC (Figs. 5 and 6). Initially calcium was released from intracellular storage at both 25 and 37°C and additional calcium increase resulted from its entry through plasma membrane channels (Figs. 1C and 2) that strongly depends on the STIM1 and Ca^{2+} -ATPase activity, which occurs at 37°C but generally reduces at 25°C (38). Increasing the membrane tension of the surrogate APC also increased the calcium levels (Fig. 3) (33).

Calcium signal was monitored using an optical path added to our micropipette experimental system. This system allows us to vary the following parameters: 1) the timing when the T cell begins and ends its contact with the pMHC-bearing RBC, 2) the speed of approach and separation between the cell pair, 3) the duration of contact, and 4) the intermission period between consecutive contacts. Using this system, for each T cell whose intracellular calcium concentration was measured over time, our temporal manipulation of its contacts with the pMHC-bearing RBC and mechanical measurement of the resulting adhesions generated a concurrent time course of formation and dissociation of TCR–pMHC and/or TCR–pMHC–CD8 bonds under force-free condition as well as bond rupture by pulling forces. By correlative analysis of these two time courses we made several observations.

First, repeated contact and separation cycles were required for calcium induction. This was apparent in that long continuous cell contact (10 min) between individual T cells and pMHC-bearing RBCs failed to trigger. Calcium was interrupted when the repeated test cycles were stopped, but resumed once cycles were restarted (Fig. 1D-F). Second, mechanical force pulling on the TCR was required for calcium induction because Ca^{2+} could not be induced by TCR–pMHC and TCR–pMHC–CD8 bonds formed and dissociated in the force-free portions of the contacts. This was shown by the lack of correlation of calcium with the adhesion frequency, the cumulative number of bonds predicted to form and the accumulation of their predicted lifetimes (Figs. 5A and C, 6A, B, D, and E, and S3).

Third, accumulation of pulling forces was required for calcium induction because the calcium signals correlated with the number of adhesions observed at the end of the repeated contact when force was applied to rupture the adhesions and independent of their duration (Fig. 6C&F). Fourth, force must be applied to TCR with sufficient frequency and duration because prolonging the intermission period between consecutive contacts (Fig. 4) or shortening the retraction time to rupture the TCR and/or CD8 bonds with pMHC (Fig. S2) decreased the calcium signals. Taken together, these data indicate that signaling via the TCR is mechanical, transient, intermittent, reversible, and cumulative.

These findings support or suggest possible extension and modification of models of T-cell activation and antigen discrimination. Our data demonstrate that, under our experimental conditions, the series of reaction steps of the signaling cascade required for calcium induction in T cells can be triggered but must be sustained by different TCRs rapidly forming and breaking bonds with agonist pMHC (with or without concurrent binding and unbinding with CD8) in a sequential manner. This emphasizes the importance of both on- and off-rates, supporting a recently extended version of the kinetic proofreading model (39, 40). The transient, intermittent, reversible, and cumulative nature of T-cell triggering indicates that intermediate signaling states would persist when the TCR–pMHC bonds dissociate and the proofreading steps would resume upon their rebinding, as proposed by the kinetic proofreading model (39, 40) and recently extended version of the serial engagement model (7). Our data suggest that the concentrations of signaling intermediates may represent the intermediate signaling states, which have to be built up progressively by sequential engagements of different TCRs. During intermission between two successive TCR–pMHC and/or TCR–pMHC–CD8 bonds, these concentrations may decrease gradually but not instantaneously (see Fig. 1E), which may provide a memory mechanism for the proofreading steps to resume upon TCR re-engagement.

Our study has provided insight to the question of how the TCR mediates mechanosensing of the T cell, showing that serial tensile forces on TCR and/or CD8 induced calcium. In a pioneering study, Kim *et al.* used optical tweezers-trapped beads bearing pMHC or anti-CD3 to engage the TCR and concluded that calcium was triggered by sinusoidal forces tangential but not normal to the cell surface (41). However, the trapped bead might rotate, potentially generating tensile forces on the TCR–pMHC and/or TCR–pMHC–CD8 bonds at the rear edge. In another study, Li *et al.* used a micropipette to aspirate the T cell and found that Ca^{2+} was triggered by both tangential and normal forces on the CD3 (42). It should be noted that the T-cell surface has a rough microtopology with numerous microvilli and ruffles (43, 44), which can be easily stretched to reorient along the direction of force (45, 46), making precise conclusions on the direction of force difficult. We have used correlative analysis between calcium signals and single TCR–pMHC bond lifetimes under force to define the nature of the TCR mechanosensor (Liu et al., unpublished data).

Intracellular calcium is a required early signaling event for activation of T cells (3-5). During their life cycle, T cells experience rich and variable mechanical microenvironments, providing ample opportunities for force to exert on the TCR, the coreceptor, and other T cell surface and cytoplasmic molecules (35). As T cells migrate and form kinapses with APCs, force should act on TCR–pMHC and/or TCR–pMHC–CD8 bonds due to relative motions

between the two cell membranes. Cell surface proteins are anchored in the membrane often with connections to actin and myosin motors that can propel retrograde flow and cyclic protrusion-contraction that occurs in the immunological synapses formation (47, 48). Of interest, several other receptor–ligand interactions on T cells key for optimal activation, including members of the integrin family, also perform better under conditions of applied force (49–51). Therefore, mechanosensing of force applied to T cell surface proteins and their receptors on interacting cells appear to be a fundamental requirement for T cell activation. Determining how mechanical force specifically regulates T-cell function will be an important goal for future studies and design of therapeutics to regulate lymphocyte activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Larissa Doudy for technical support, Kaitao Li for helping the VSV control experiment, and the NIH Tetramer Facility for providing the pMHCs

This work was supported by NIH grants AI38282 and GM096187 (to C.Z.) and AI096879 (to B.E.).

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Abbreviations used in this article

2D	two-dimensional
3D	three-dimensional
SPR	surface plasmon resonance
AUC	area under the curve

Figure 1. Concurrent adhesion frequency and calcium imaging experiment

A. A RBC (left cell) was driven rightward (arrow) to contact an OT1 T cell (right cell) to prompt adhesion (upper left panel), which was detected by RBC elongation (lower left panel) upon retraction (arrow). Adhesion (red) and no adhesion (gray) events were recorded as 1's and 0's and their running average is plotted vs. experimental time (right panel) for OVA (upper curve) or VSV (lower curve) pMHC. 2D parameters measured from the upper curve are indicated (see text for definitions). **B.** Representative pseudocolor images showing intracellular calcium levels at indicated times while the T cell was being repeatedly touched by a biotinylated RBC coated with VSV (top row) or OVA (middle row) pMHC or without pMHC (bottom row). **C.** Calcium time courses (mean $\pm$ SEM of 12 cell pairs) in OT1 T cells induced by repeated contacts with biotinylated RBCs coated with 16 OVA:H-2K^b/μm² at two temperatures. The two overlapping negative control curves respectively generated by biotinylated RBCs coated with 24VSV:H-2K^b/μm² and without coating define the basal calcium change in our experimental system. All calcium curves were normalized by their respective initial values at 0 min. **D** and **E.** Calcium traces in individual T cells contacted by RBCs bearing 16 OVA:H-2K^b/μm² in repeated cycles for 10 min (green) or only the first 3 min (black) (**D**) or for the first 200 s, stopped for 200 s, and either resumed (green) or did not resume (black) the repeated cycles for the remaining time (**E**). **F.** Calcium traces of 2 T

cells that were continuously contacted by RBCs bearing 16 OVA:H-2K^b/μm² without separation for 10 min.

Figure 2. Quantification of calcium response and two sources of calcium release

A. Strong correlation between I_{max} and AUC. To reduce data representation, the variable calcium curve in a 10-min time course was quantitated either by the maximal calcium level (normalized by the initial value), I_{max} , or by the area under the normalized calcium curve, AUC. The I_{max} vs. AUC plot demonstrates a strong linear correlation ($R^2=0.975$). **B.** Two sources of calcium release. Calcium in T cells was measured at 25 and 37°C in the presence of 2 mM extracellular calcium or 1mM EGTA in the media during cyclic contacts by biotinylated RBCs coated with 16 OVA:H-2K^b/μm². Data were presented as mean ± SEM of AUC of a 10-min time course for 12 T-cell–RBC pairs from >3 experiments.

Figure 3. Dependence of calcium signal on RBC membrane tension

A-C Representative calcium traces showing triggering (black) and non-triggering (gray) examples for the three tension regimes defined in panel D. **D.** Scattergram of the maximal Fura-2 ratio $I_{\max}$ (i.e., highest relative Fura-2 ratio of the 10-min calcium curve compared to the initial level) vs. membrane tension for 82 OT1 T cells each tested by a RBC coated with 20 OVA:H-2K^b/μm². Linear regression (dashed line) to individual cell data (open circles) show significant non-zero linear slope ($p < 0.05$, F-test). Linear fit (solid line) to the averaged values in the divided tension regimes (mean ± SEM, closed circles) shows a steeper slope with a good correlation ($R^2 = 0.92$). As the membrane tension increased across the three regimes, the percent of T cells showing significant calcium increase (defined as $I_{\max} > 1.2$) rose from 15 to 23 and 30%. We expect similar conclusions should calcium increase be measured by another metric, e.g., AUC since $I_{\max}$ and AUC strongly correlate (Fig. 2A).

Figure 4. Frequently applied force pulling on TCR and/or CD8 by engaged agonist pMHC is required for sustain calcium signal

Each T-cell–RBC pair was tested repeatedly for 2 s contact duration and varied pause periods between consecutive contacts (0, 5 and 10 s). **A.** Voltage waveforms used to drive the repeated approach and retraction movements of the RBC pipette. “1” and “0” represent, respectively, contact and no contact between RBC and T cell. Fura-2 ratiometric images were captured every 2 seconds. **B** and **C.** Mean $\pm$ SEM (of three measurements each curve) of Fura-2 ratios (normalized to the respective initial values) induced by repeated cycles of the indicated waveforms in OT1 T cells contacted by RBCs coated with 18.5 OVA:H-2K^b/μm² (**B**) or 22 A2:H-2K^b/μm² (**C**).

Figure 5. Comparing contact duration-dependent calcium level with adhesion parameters
A. Two- and single-stage patterns of P_a vs. t_c plots for OT1 T cells interacting with RBCs coated with 14.7 OVA:H-2K^b/μm² or 27.7 OVA:H-2K^bα3A2/μm², respectively. Nonspecific adhesion to biotinylated RBCs without ligand coating was negligibly low. So was binding to RBCs coated with 24 VSV:H-2K^b/μm², in agreement with our previous finding that without cooperation with the TCR, CD8 binding is undetectably low at such low pMHC density (25). For each t_c , mean $P_a \pm$ SEM was measured from 10-12 cell pairs each tested by repeated cycles for 10 min. **B.** Mean $\pm$ SEM of AUC is plotted vs. t_c for OT1 T cells tested by repeated contact cycles for 10 min with RBCs coated with H-2K^b or H-2K^bα3A2 bound with OVA at 37°C or 25°C. **C.** Numbers of bonds N_b formed during each contact of a given t_c and their lifetimes t_b were calculated using P_a of each cell pair and the previously measured off-rate according to Materials and Methods (cf. Eqs. 5b and 6b, respectively). Their respective accumulations, ΣN_b and Σt_b , over the entire 10-min experimental period were calculated from 10-12 cell pairs as mean $\pm$ SEM and plotted vs. t_c . **D.** Adhesion events were enumerated from 10-min repeated cycles of a given t_c for OT1 T cells contacting RBCs coated with OVA:H-2K^b or OVA:H-2K^bα3A2, respectively. Biotinylated RBCs coated with VSV:H-2K^b or without ligand coating serve as negative controls for nonspecific adhesion. The mean $\pm$ SEM values from 10-12 cell pairs were plotted vs. t_c .

Figure 6. Correlating calcium with adhesion parameters

Mean $\pm$ SEM of the area under the 10-min Fura-2 fluorescence ratio time course (AUC) (A-C) or maximal Fura2 ratio (I_{max}) (D-F) was measured by live-cell imaging in 12-15 OT1 T cells repeatedly contacted by RBCs coated with OVA:H-2K^b or OVA:H-2K^bα3A2 for 10 min at a given contact duration ranging from 0.25-10 s and plotted vs. the adhesion frequency P_a (A and D), cumulative number of bonds predicted to form during all repeated contacts ΣN_b (B and E, upper x-axis), the predicted cumulative lifetimes of these bonds Σt_b (B and E, lower x-axis), or the total number of adhesion events observed N_a (C and F). The corresponding contact durations used to obtain these data are indicated by the numbers (in second) next to the points