Accumulation of Serial Forces on TCR and CD8 Frequently Applied by Agonist Antigenic Peptides Embedded in MHC Molecules Triggers Calcium in T Cells

Sergey Pryshchep, Veronika I. Zarnitsyna, Jinsung Hong, Brian D. Evavold and Cheng Zhu

*J Immunol* 2014; 193:68-76; Prepublished online 2 June 2014;
doi: 10.4049/jimmunol.1303436
http://www.jimmunol.org/content/193/1/68

Supplementary Material

http://www.jimmunol.org/content/suppl/2014/05/31/jimmunol.1303436.DCSupplemental

References

This article cites 51 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/193/1/68.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Accumulation of Serial Forces on TCR and CD8 Frequently Applied by Agonist Antigenic Peptides Embedded in MHC Molecules Triggers Calcium in T Cells

Sergey Pryshchep,*1 Veronika I. Zarnitsyna,*2 Jinsung Hong,† Brian D. Evavold,‡ and Cheng Zhu,*†

T cell activation by Ag is one of the key events in adaptive immunity. It is triggered by interactions of the TCR with peptide epitopes embedded in MHC (pMHC) molecules expressed on APCs. The mechanism of how signal is initiated remains unclear. In this article, we complement our two-dimensional 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 applications impaired calcium signals. Our data support a model where rapid accumulation of serial forces on TCR–pMHC–CD8 bonds triggers calcium in T cells. The Journal of Immunology, 2014, 193: 68–76.

T cell effector functions result from activation of signaling cascades triggered by interactions of the TCR with antigenic peptides embedded in MHC (pMHC) (1, 2). TCR–pMHC interaction in cooperation with coreceptor CD4 or CD8 initiates phosphorylation of the ITAMs of the CD3 by the lymphocyte-specific protein tyrosine kinase p56lck, which allows docking of the ζ-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 phospholipase Cγ (1). This initiates the production of diacylglycerol and inositol-1,4,5-trisphosphate, which increases 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).

Because TCR is the only molecule on the T cell surface that interacts with specific Ag, 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 measurements are found to correlate with T cell activation better than their three-dimensional 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 two-dimensional kinetic measurement. To address this shortcoming, we combined the high-temporal-resolution micropipette two-dimensional kinetic analysis with concurrent calcium imaging in this study, because 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

Naïve CD8+ T cells from OT1 transgenic mice were obtained using an Emory University Institutional Animal Care and Use Committee–approved protocol. The following peptides were synthesized: OVA-derived peptides OVA (SIINFEKL), A2 (SAINFEKL), G4 (SIIGFEKL), E1 (EINFEKL), and R4 (SIRFEKL), and a vesicular stomatitis virus (VSV)–derived peptide VSV (RGYYVYGL) (7). OVA, A2, E1, and R4 are recognized by the OT1 TCR, but VSV is a null peptide. Monomeric mouse pMHC-I H-2Kb with C-terminal biotin tags and pMHC-I mutant (H-2KbΔ32A) were produced by the National Institutes of Health Tetramer Core Facility.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1303436

Copyright © 2014 by The American Association of Immunologists, Inc. 0022-1767/14/$16.00
PE-conjugated anti-mouse TCR Vα2 (B20.1) and VB 11 (RR3-15) were from BD Biosciences (San Jose, CA), Anti-mouse H-2Kb (3H2672, PE-conjugated) was from US Biological (Swampscott, MA) and Biocarta (San Diego, CA), respectively. Anti-biotin (BioZ-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 Institutional Review Board–approved protocol as described previously (7). RBCs were biotinylated with Biotin-X-NHS (EMD) at different concentrations as described previously (24), to coat different pMHC densities. 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 previously (7).

Determining RBC membrane tension
RBC membrane tension (TRBC) was determined from the suction pressure (∆P) and the respective radii of the micropipette (Rp) and the spherical portion of the RBC outside the pipette (Rw) using the Laplace equation, which results in the following equation (30):

\[ TRBC = \frac{\Delta P}{2} \left( \frac{1}{R_p} + \frac{1}{R_w} \right) \]  

(Dual micropipette system for two-dimensional kinetic analysis)
We customized an inverted microscope (TMD Diaphot; Nikon) by mounting two identical sets of three-dimensional 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 LVPTZ (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 simultaneously observed them through a TV monitor and recorded by a VCR.

The micropipette adhesion frequency assay has been described previously (20, 21). In brief, a T cell and an RBC were aspirated by respective pipettes (Fig. 1) and driven to contact with controlled area Ac and duration tc. 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. Ten to 12 cell pairs were used to estimate an adhesion frequency PA (mean ± SEM) at each given contact duration, which was calculated as the number of adhesions Na divided by the number of total contacts Nc.

Calculations of the number of bonds and their lifetimes accumulated over the 10-min experimental period
The average number of bonds (n) present at any time during a contact can be calculated from the adhesion frequency PA (7, 20):

\[ n = \frac{-P_A}{ln(1 - P_A)} \]  

Because of the rapid two-dimensional kinetics of the OT1 TCR–OVA: H-2Kb×3A2 interaction (Fig. 4A) (7), at contact durations tc > 0.1 s, (n) reached steady-state and is equal to:

\[ n = m_A \cdot K_A \]  

where m and K are the respective site densities of the TCR and pMHC, and the product A · K is the contact area and K is the two-dimensional affinity. The effective two-dimensional affinity (7, 20). Eq. 3 is obtained by setting tc = ∞ in equation 2 of the Supplemental Methods of Ref. 7. Because koff ranges from 1 to 10 s−1 (7), a few seconds of contact time tc is long enough to be considered as infinity because exp(−koff · tc) would be negligible compared with 1. It follows from Eq. 3 that multiplying (n) by the previously measured two-dimensional off rate koff (10 s−1) yields the cellular rate on, or the frequency of bond formation during T cell–RBC contact:

\[ m_A \cdot K_A \cdot k_{on} = m_A \cdot K_A \cdot K_B \times k_{off} = (n) \times k_{off} \]  

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

Na = (n) × koff × tc \hspace{1cm} (5a)

\[ \Sigma N_a = (n) \times koff \times \Sigma t_c = \Sigma (n) \times koff \times t_c \]  

Because the reciprocal off rate is the average bond lifetime (31), we can multiply Na or ∑Na by 1/koff to obtain the length of time td during the contact when a TCR or TCRs are engaged with pMHC (or the cumulative bond lifetime ∑Na):

\[ t_d = N_a / koff = (n) \times t_c \]  

\[ \Sigma t_d = \Sigma N_a / koff = (n) \times \Sigma t_c \]  

(6a)

Thus, (n) is also the fraction of contact time during which the TCR is engaged with pMHC. The calculations for the case of TCR–OVA.H-2Kb–CD8 trimeric interaction are assumed similar.

Calcium measurements
Naive CD8+ T cells were loaded with Fura-2/AM (3 μM in DMSO), incubated for 30 min at 37°C, and washed twice with PBS buffer (pH 7.4). T cells and RBCs precoated with pMHC were transferred into microscopic chamber with L-15/HEPES media (Sigma). Similar to the previously used adhesion frequency assay for two-dimensional 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 were performed using NIS-Element software (Nikon). For chelating extracellular Ca2+, EGTA (final concentration 1 mM) was injected directly into chamber medium. Experiments were done at 37°C unless otherwise stated.

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

Results
We upgraded our micropipette system by adding an optical path to enable concurrent micromanipulation and calcium imaging (Supplemental Fig. 1). The micropipette adhesion frequency assay (20) uses an RBC as a surrogate APC for presentation of H-2Kb 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 naive OT1 CD8+ T cell (Fig. 1A, lower panel). The presence of a bond or bonds at the end of a given contact duration tc is registered by RBC elongation upon its retraction (Fig. 1A, lower left panel), which pulls on the TCR and/or CD8 via the previ-
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 ($I_{\text{max}}$) 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˚C 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 with 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°C 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) (30). Consistent with the previous report (33), we observed that increasing the RBC membrane tension (increased suction pressure) induced higher calcium levels (Fig. 3).

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 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 mechanism for mechanosensing.

FIGURE 2. Quantification of calcium response and two sources of calcium release. (A) Strong correlation between $I_{\text{max}}$ 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), $I_{\text{max}}$, or by the area under the normalized calcium curve (AUC). The $I_{\text{max}}$ versus 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°C and 37°C in the presence of 2 mM extracellular calcium or 1 mM EGTA in the media during cyclic contacts by biotinylated RBCs coated with 16 OVA-H-2Kb/m2. 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 nontriggering (gray) examples for the three tension regions defined in (D). (D) Scattergram of the maximal Fura-2 ratio $I_{\text{max}}$ (i.e., highest relative Fura-2 ratio of the 10 min calcium curve compared with the initial level) versus membrane tension for 82 OT1 T cells each tested by a RBC coated with 20 OVA-H-2Kb/m2. Linear regression (dashed line) to individual cell data (open circles) shows significant nonzero slope ($p < 0.05$, F-test). Linear fit (solid line) to the averaged values in the divided tension regions (mean ± SEM, closed circles) shows a steeper slope with a good correlation ($R^2 = 0.92$). As the membrane tension increased across the three regions, the percent of T cells showing significant calcium increase (defined as $I_{\text{max}} > 1.2$) increased from 15 to 23 and 30%. We expect similar conclusions should calcium increase be measured by another metric, for example, AUC, because $I_{\text{max}}$ and AUC strongly correlate (Fig. 2A).
in T cells (Supplemental Fig. 2). 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 trigger 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 RBCs, 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, whereas 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 sharply increased to a second plateau, which was CD8 and/or TCR–pMHC–CD8 interactions and imaging of intracellular 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˚C 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 Ag recognition by a T cell (38). 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 (Supplemental Fig. 3A–C). The variant agonist A2 induced a similar AUC versus \( t_c \) curve (Supplemental Fig. 3D) 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 (Supplemental Fig. 3E and 3F), 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 Ag 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

**FIGURE 4.** Frequently applied force pulling on TCR and/or CD8 by engaged agonist pMHC is required for sustaining calcium signal. Each T cell–RBC pair was tested repeatedly for 25 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 s. (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\(^b\)/\( \mu \)m\(^2\) (B) or 22 A2:H-2K\(^b\)/\( \mu \)m\(^2\) (C).
correlative analysis of the two time courses. We chose AUC and the maximal Fura-2 ratio $I_{\text{max}}$ as the metrics for analyzing the calcium time course. To reduce data representation of the repeated contact cycles, we calculated the adhesion frequency $N_a$, the cumulative number of bonds $\sum N_b$ predicted to form in all contacts (cf. Eq. 5b), the accumulation of bond lifetime $\sum t_b$ during all contacts (cf. Eq. 6b) when a TCR or TCRs are predicted to engage with pMHC with or without the coengagement of CD8, and the number of observed adhesions $N_c$. Note that $\sum N_b$ and $\sum t_b$ represent, respectively, the total number of bonds formed and the accumulation of their lifetimes during all cyclic contacts $\sum t_b$ 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. Therefore, $N_c$ 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 $\sum N_b$ (Fig. 5C, left ordinate) versus $t_c$, $\sum t_b$ (Fig. 5C, right ordinate) versus $t_c$, and $N_a$ versus $t_c$ (Fig. 5D) curves with the AUC versus $t_c$ curve (Fig. 5B). The cumulative number of bonds $\sum N_b$ and their cumulative lifetimes $\sum t_b$ 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 $N_c$ for OVA:H-2K$^\beta$ increased with $t_c$ when $t_c \leq 1$ s, but decreased after $t_c \approx 2$ 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 $t_c \approx 2$ s. It is telling that the AUC versus $t_c$ curves (Fig. 5B) do not resemble the $P_a$ (Fig. 5A), $\sum N_b$, and $\sum t_b$ (Fig. 5C) versus $t_c$ curves, but resemble the $N_a$ versus $t_c$ curves (Fig. 5D). Using $I_{\text{max}}$ instead of AUC as calcium induction metric (Supplemental Fig. 4), 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 versus $P_a$, $\sum N_b$, $\sum t_b$, 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 and 5C, repeat contacts by OVA:H2-K$^\beta$ induced intracellular calcium fluxes in OT1 T cells that first increased with increasing $P_a$, $\sum N_b$, and $\sum t_b$ (Fig. 6A, 6B, 6D, and 6E). After reaching a maximum, $Ca^{2+}$ rapidly decreased to the level comparable with that of OVA:H2-K$^\beta$α3A2, which remained very low. This calcium decrease in response to increasing $P_a$, $\sum N_b$, and $\sum t_b$ 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 and 6F). 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 $t_c \geq 2$ s where

$\text{FIGURE 5.}$ Comparing contact duration–dependent calcium level with adhesion parameters. (A) Two- and single-stage patterns of $P_a$ versus $t_c$ plots for OT1 T cells interacting with RBCs coated with 14.7 OVA:H-2K$^\beta$μm$^2$ or 27.7 OVA:H-2K$^\beta$α3A2/μm$^2$, respectively. Non-specific adhesion to biotinylated RBCs without ligand coating was negligibly low; so was binding to RBCs coated with 24 VSV:H-2K$^\beta$μm$^2$, in agreement with our previous finding that without cooperation of 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 versus $t_c$ for OT1 T cells tested by repeated contact cycles for 10 min with RBCs coated with H-2K$^\beta$ or H-2K$^\beta$6 coated with OVA:H-2K$^\beta$ or OVA:H-2K$^\beta$6, respectively. Their respective accumulations, $\sum N_b$ and $\sum t_b$ over the entire 10-min experimental period were calculated from 10–12 cell pairs as mean $\pm$ SEM and plotted versus $t_c$. (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$^\beta$ or OVA:H-2K$^\beta$α3A2, respectively. Biotinylated RBCs coated with VSV:H-2K$^\beta$ or without ligand coating serve as negative controls for non-specific adhesion. The mean $\pm$ SEM values from 10–12 cell pairs were plotted versus $t_c$. 

$P_{\alpha}$ represents a small fraction of the $\sum N_b$ (Fig. 5A, left ordinate) versus $t_c$, $\sum t_b$ (Fig. 5A, right ordinate) versus $t_c$, and $N_a$ versus $t_c$ (Fig. 5D) curves with the AUC versus $t_c$ curve (Fig. 5B). The cumulative number of bonds $\sum N_b$ and their cumulative lifetimes $\sum t_b$ 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 $N_c$ for OVA:H-2K$^\beta$ increased with $t_c$ when $t_c \leq 1$ s, but decreased after $t_c \approx 2$ 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 $t_c \approx 2$ s. It is telling that the AUC versus $t_c$ curves (Fig. 5B) do not resemble the $P_a$ (Fig. 5A), $\sum N_b$, and $\sum t_b$ (Fig. 5C) versus $t_c$ curves, but resemble the $N_a$ versus $t_c$ curves (Fig. 5D). Using $I_{\text{max}}$ instead of AUC as calcium induction metric (Supplemental Fig. 4), 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 versus $P_a$, $\sum N_b$, $\sum t_b$, 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 and 5C, repeat contacts by OVA:H2-K$^\beta$ induced intracellular calcium fluxes in OT1 T cells that first increased with increasing $P_a$, $\sum N_b$, and $\sum t_b$ (Fig. 6A, 6B, 6D, and 6E). After reaching a maximum, $Ca^{2+}$ rapidly decreased to the level comparable with that of OVA:H2-K$^\beta$α3A2, which remained very low. This calcium decrease in response to increasing $P_a$, $\sum N_b$, and $\sum t_b$ 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 and 6F). 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 $t_c \geq 2$ s where...
the adhesion frequency curve is in the second plateau (Fig. 5A). Contacts at threshold duration $t_c = 1$ s produced both bimolecular and trimolecular bonds, therefore inducing an intermediate level of calcium that connected the two segments. The reverse relationship between $N_b$ and $t_c$ is evident from Fig. 6C and 6F, explaining the decrease in calcium with increasing $t_c$ as it exceeded 2 s (Fig. 5B and Supplemental Fig. 3). It also explains the calcium decrease in Fig. 6B and 6E when $\sum t_b > 3500$ ($\sum n_b > 350$ s), because these correspond to increasing $t_c$ and decreasing $N_b$. 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 article, we studied calcium responses to pMHC engagement with TCR with and without the coengagement 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. 1C and 2) that strongly depends on the stromal interaction molecule 1 and Ca$^{2+}$–ATPase activity, which occurs at 37°C but generally reduces at 25°C (39). 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 surrogate APC also increased the calcium levels (Fig. 3) (33).

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 conditions, as well as bond rupture by pulling forces. By correlating 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 with the calcium and adhesion frequency, the cumulative number of bonds predicted to form, and the accumulation of their predicted lifetimes (Figs. 5A, 5C, 6A, 6B, 6D, 6E, and Supplemental Fig. 3). 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 and 6F). 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 (Supplemental Fig. 2) 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 Ag 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 (40, 41). 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 (40, 41) 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. (42) used optical tweezers-trapped beads bearing pMHC or anti-CD3 to engage the TCR and concluded that calcium was triggered by sinuousoidal forces tangential but not normal to the cell surface. 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. (43) used a micropipette to aspirate the T cell and found that Ca²⁺ was triggered by both tangential and normal forces on the CD3. It should be noted that the T cell surface has a rough microtopology with numerous microvilli and ruffles (44, 45), which can be easily stretched to reorient along the direction of force (46, 47), 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 (37).

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 because of 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 (48, 49). Of interest, several other molecular interactions on T cells key for optimal activation, including actin and members of the integrin family, also perform better under conditions of applied force (50–52). 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.

Acknowledgments
We thank Larissa Doudy for technical support, Kaitao Li for help with the VSV control experiment, and the National Institutes of Health Tetramer Facility for providing pMHCs.

Disclosures
The authors have no financial conflicts of interest.

References


**Supplemental Figure 1. Concurrent micromanipulation and calcium imaging system.** An inverted microscope (TMD Diaphot, Nikon) was customized by mounting two sets of mechanical manipulators on the two sides of the stage. A piezo actuator is mounted on one side to drive micromanipulation of glass pipette for adhesion frequency assay. Concurrent detection of adhesion frequency and calcium signaling is enabled by splitting the bright field light and Fura-2 excitation wave lengths. Decoupling the bright field and fluorescent images is obtained by using 610-nm long-pass filter for bright field light and DC2 system with 565 nm long-pass dichroic mirror for fluorescent imaging.
Supplemental Figure 2. Calcium increased with decreasing pulling rate. Calcium traces from individual T cells during 10 min of contact-retract cycles with RBCs coated with 16 OVA:H-2K\textsuperscript{b}/μm\textsuperscript{2} at different retraction speed. The data are expressed as fold changes in the Fura-2 ratio from the starting time point.
Supplemental Figure 3. High densities of antagonist pMHCs bound T cells with similar adhesion frequencies to agonist pMHCs but failed to trigger calcium. Adhesion frequency $P_a$ vs. contact time $t_c$ curves were measured for OT1 T cells interacting with RBCs coated with 22 A2:H-2K$^b$/µm$^2$ (A), 1240 E1:H-2K$^b$/µm$^2$ (B), or 1490 R4:H-2K$^b$/µm$^2$ (C) similar to Fig. 5. Biotinylated RBCs coated with 24 VSV:H-2K$^b$/µm$^2$ and/or without coating define as negative control in our experimental system. For each points of contact duration, the average calcium response was measured as the Area Under the Curve (AUC) from more than three independent experiments (D and F). Data are shown as average ± SEM.
**Supplemental Figure 4. Dependence of calcium level on contact duration.** Maximum percent increase in Fura-2 ratio relative to the initial value ($I_{\text{max}}$, mean ± SEM) is plotted vs. $t_c$ for OT1 T cells tested by 10-min repeated cycles with RBCs coated with 14.7 H-2K$^b$/µm$^2$ (○△) or 27.7 H-2K$^b$α3A2/µm$^2$ (●▲) bound with OVA at 37°C (○●) or 25°C (△▲).