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 agonist peptide 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.
PE-conjugated anti-mouse TCR Vα2 (B20.1) and Vβ11 (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 (BioX-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 (R0) and the spherical portion of the RBC outside the pipette (RBRBC) using the Law of Laplace, which results in the following equation (30):

\[ T_{RBC} = \frac{\Delta P}{2} \left( \frac{1}{R_0} - \frac{1}{R_{RBC}} \right) \]  

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 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 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 Pn (mean ± SEM) at each given contact duration, which was calculated as the number of adhesions Nn 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 Pn (7, 20):

\[ n = -\ln(1 - P_n) \]  

Because of the rapid two-dimensional kinetics of the OT1 TCR–OVA: H-2Kbα32A2 interaction (Fig. 4A) (7), at contact durations tc > 0.1 s, (n) reached steady-state and is equal to:

\[ \langle n \rangle = m_n m_A K_n \]  

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

\[ m_n m_A K_n \times k_{off} = (n) \times k_{off} \]  

Multiplying the frequency of bond formation (Eq. 4) by a given contact duration tc (or the cumulative contact duration \( t_{cum} \) for the entire 10-min experiment) yields the number of bonds formed at that contact \( N_n \) (or the cumulative number of bonds formed \( \sum N_n \)).

\[ N_n = (n) \times k_{off} \times t_c \]  

\[ \sum N_n = (n) \times k_{off} \times \sum t_c = \sum (n) \times k_{off} \times t_c \]  

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

\[ t_n = N_n / k_{off} = (n) \times \sum t_c \]  

\[ \sum t_n = \sum N_n / k_{off} = (n) \times \sum t_c \]  

Thus, \( \langle n \rangle \) is also the fraction of time during which the TCR is engaged with pMHC. The calculations for the case of TCR–OVA:H-2Kb–CD8 trimolecular 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 micropipette 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, left panels). The presence of a bond or bonds at the end of a given contact duration \( t_n \) is registered by RBC elongation upon its retraction (Fig. 1A, lower left panel), 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 \( P_n \) is calculated as the number of adhesions \( N_n \) 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 RBCs induced calcium in T cells

Repeated formation and detachment of T cell adhesions to RBCs coated with OVA:H-2Kb induced a specific increase in intracellular calcium that reached a maximum level within 4–5 min at 37˚C (Fig. 1B, middle row, and 1C, green curves). 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-2Kb instead of OVA:H-2Kb was coated on biotinylated RBCs or when no pMHC was coated (Fig. 1B bottom row and Fig. 1C light blue dashed curve). Cyclic T cell contacts with surrogate APCs were required because 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 ($I_{\text{max}}$) 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). To achieve the piconewton sensitivity of the force transducer with a soft RBC, yet trigger good levels of calcium, we used midrange membrane tensions for all experiments in this article 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, 37). 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 (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 ($\sim$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 dependent by TCR–pMHC–CD8 trimolecular interaction as shown using the mutant MHC (H-2K$^b$/α3A2) 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, because it was not observed using H-2K$^b$/α3A2. 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 a single bond is the subject of another publication (37). Using the mutant MHC to prevent CD8 binding abolished calcium response at 25°C, whereas 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°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...
OT1 T cells interacting with RBCs coated with 14.7 OVA:H-2Kβ give without cooperation with the TCR, CD8 binding is undetectably low at such low pMHC density (25). For each 10 min with RBCs coated with H-2Kβ or H-2Kβ for nonspecific adhesion. The mean coated with OVA:H-2Kβ or OVA:H-2Kβ is reduced by increasing contact duration per cycle beyond t0. It is telling that the AUC versus t0 is not significant (Fig. 5B). By comparison, adhesions were observed only when a bond or bonds (if any) were pulled by force (Fig. 1A).

The correlational analysis was first done by comparing the ∑Nc bonds (Fig. 5C, left ordinate) versus tc (Fig. 5B), ∑nb versus tc (Fig. 5C, right ordinate), and ∑na versus tc (Fig. 5D) curves with the AUC versus tc curve (Fig. 5B). The cumulative number of bonds ∑Nc and their cumulative lifetimes ∑tc increased monotonically with the contact duration tc (Fig. 5C), as expected from their respective definitions (cf. Eqs. 5b and 6b). The number of observed adhesions Nc, Nn, and Nna represent, respectively, the total number of bonds formed and the accumulation of their lifetimes during all cyclic contacts ∑tc in the absence of tensile force (20). By comparison, adhesions were observed only at the end of tc when a bond or bonds (if any) were pulled by force to stretch the RBC. Therefore, Nc represents a small fraction of the total bonds formed that were pulled by force (cf. Fig. 1A).

The correlational analysis was first done by comparing the ∑Nc (Fig. 5C, left ordinate) versus tc (Fig. 5B), ∑nb (Fig. 5C, right ordinate) versus tc, and ∑na versus tc (Fig. 5D) curves with the AUC versus tc curve (Fig. 5B). The cumulative number of bonds ∑Nc and their cumulative lifetimes ∑tc increased monotonically with the contact duration tc (Fig. 5C), as expected from their respective definitions (cf. Eqs. 5b and 6b). The number of observed adhesions Nc for OVA:H-2Kβ increased with tc when tc ≤ 1 s, but decreased after tc ≥ 2 s (Fig. 5D). This is because Pn increased with tc before the second-stage adhesion (see Fig. 5A), but the total number of contact cycles repeated in the 10-min experiment, Nc = Nc/Pn, was reduced by increasing contact duration per cycle beyond tc ≥ 2 s. It is telling that the AUC versus tc curves (Fig. 5B) do not resemble the Pn (Fig. 5A), ∑Nn, and ∑nb (Fig. 5C) versus tc curves, but resemble the Nc versus tc curves (Fig. 5D). Using Πmax 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 Pn, ∑Nn, ∑nb, and Nc 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:H-2Kβ induced intracellular calcium fluxes in OT1 T cells that first increased with increasing Pn, ∑Nn, and ∑nb (Fig. 6A, 6B, 6D, and 6E). After reaching a maximum, Ca2+ rapidly decreased to the level comparable with that of OVA:H-2Kβ, which remained very low. This calcium decrease in response to increasing Pn, ∑Nn, and ∑nb refuted the adhesion frequency, cumulative number of bonds, and their cumulative lifetimes as determining parameters of calcium signals. By comparison, Ca2+ increased with Nc 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 tc ≤ 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 tc ≥ 2 s where

**FIGURE 5.** Comparing contact duration–dependent calcium level with adhesion parameters. (A) Two- and single-stage patterns of Pn versus tc plots for OT1 T cells interacting with RBCs coated with 14.7 OVA:H-2Kβ or 27.7 OVA:H-2Kβ:α3A2, respectively. Nonspecific adhesion to biotinylated RBCs without ligand coating was negligibly low; so was binding to RBCs coated with 24 VSV:H-2Kβ or VSV:H-2Kβ:α3A2, respectively. Biotinylated RBCs coated with VSV:H-2Kβ or without ligand coat serve as negative controls for nonspecific adhesion. The mean ± SEM values from 10–12 cell pairs were plotted versus tc.
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 $N_c$ 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 $\sum N_b > 3500$ ($\sum N_t > 350$ s), because these correspond to increasing $t_c$ and decreasing $N_c$. 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. 5 and 6). Initially, calcium was released from intracellular storage at both 25°C and 37°C, and additional calcium increase resulted from its entry through plasma membrane channels (Figs. 1C and 2) that strongly depends on the stromal interaction molecule 1 and Ca²⁺–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 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 Ca²⁺ 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, 5C, 6A, 6B, 6D, 6F, 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 re-binding, 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 sinusoidal 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 Ca++ was triggered by both tangential and normal forces on the CD3. It should be noted that the T cell surface has a rough morphotopology 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 forces to exert on the TCR, the co-receptor, 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 Tetrramer 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 enable 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^b/μm^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 ($I_{max}$, 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$^2$ (○Δ) or 27.7 H-2K$^b$α3A2/µm$^2$ (●▲) bound with OVA at 37°C (○●) or 25°C (Δ▲).