T cell activation requires force generation

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Introduction

Here, we study the mechanical forces generated by T cells undergoing activation. T cell receptors (TCRs) are triggered upon interaction with their cognate peptides presented in the major histocompatibility complex (pMHC). Recent work has suggested that triggering requires a force upon the TCR through shear (Li et al., 2010) or pulling (Liu et al., 2014). The origin of these forces is as yet unknown, but the fact that TCR triggering occurs while T cells interact with inert objects such as antibody-coated beads suggests that the major contribution of force on the TCR comes from the T cell itself. Thermal forces can alter membrane shape to induce transient, solitary receptor–ligand contacts (Lee et al., 2003), whose binding energy can lead to further membrane apposition and further TCR triggering (James and Vale, 2012). The TCR could also be exposed to shear forces when T cells and APCs move relative to one another while interacting (Beemiller and Krummel, 2010).

After initial triggering, T cells can enhance contact with antigen-presenting cells (APCs) by actively pushing actin-rich lamellipodia and invadopodia into APCs (Negulescu et al., 2009; Simonsen et al., 2009; Ma and Finkel, 2010; Puech et al., 2011) but has not been used to deliver antigenic signals to living cells except in our prior work with mast cells (Hu et al., 2014). Here, AFM allowed us to spatiotemporally control the delivery of TCR ligands while simultaneously measuring the T cell’s biochemical and mechanical responses.

Results and discussion

We coated the AFM cantilever tip with molecules to stimulate T cells (anti-CD3 or pMHC; Fig. 1 A). To ensure stable attachment of proteins to the cantilever tip, we used the chemical cross-linker sulfo-succinimidyl 6-(3′-(2-pyridyldithio)propionamido) hexanoate (sulfo-LC-SPDP) to covalently attach streptavidin to a 3-mercaptopropyl trimethoxysilane–coated silicon cantilever (Fig. S1). Fluorescence imaging of phycoerythrin-labeled streptavidin showed that the tip was well coated (Fig. 1 B). Either biotinylated pMHC or biotinylated anti-CD3 was then added to the streptavidin-coated cantilever tip. We used a newly functionalized cantilever to interrogate each cell.

Primary effector CD4+ T cells were obtained from TCR-transgenic OT-II mice, which recognize the ovalbumin peptide (323–339) in the context of I-Aβ. We measured Ca2+ influx as a proximal readout for TCR triggering, measured by change in fluorescence intensity of the Ca2+-sensitive dye Fluo-4. By gently touching the T cell surface with the anti-CD3–coated cantilever with a trigger force of 250 pN, we ligated TCR–CD3 complexes and monitored Fluo-4 intensity changes.
by fluorescence microscopy. With the cantilever in continuous contact, we observed pronounced Ca\(^{2+}\) flux (Fig. 1, C, D, and I). We also engaged T cells with cognate pMHC-coated cantilevers and observed a similar Ca\(^{2+}\) flux time profile and integrated intensity (Fig. 1, E, F, and I). In contrast, continuous contact with cantilevers coated with irrelevant monoclonal antibodies produced no significant Ca\(^{2+}\) flux (Fig. 1, G–I). For control mAbs in this work, we used both anti-CD43 and anti-CD25, which bound to cell-surface receptors (seen upon disengagement of the cantilever from the cell; not depicted) but would not be expected to activate T cells (Bunnell et al., 2002; Hosseini et al., 2009). To ensure that the increase in Fluo-4 intensity observed was truly indicative of Ca\(^{2+}\) entry and not shape changes in the T cell or changes in the focal plane upon contact with the AFM cantilever, we loaded the T cells with both Fluo-4 and Fura-Red dyes. We calculated the ratio of Fluo-4 to Fura-Red intensities and observed a similar time profile, indicating antigen-dependent Ca\(^{2+}\) flux (Fig. S2, A and B). These results indicate that AFM can deliver antigenic stimulation to T cells.

By monitoring the deflection of the AFM cantilever during contact with these same cells, we measured forces generated during activation. During continuous TCR triggering, the T cells exhibited large pushing and pulling forces on the cantilever (Fig. 2 A). In contrast, cells contacted with control mAb–coated cantilevers yielded no significant force generation (Fig. 2 B). We found that both pushing forces (Fig. 2 C) and pulling forces (Fig. 2 D) were higher in cells contacted with anti-CD3 than with control mAb. This result indicates that continuous TCR triggering results in the generation of forces by the T cell. T cells pushed against pMHC-coated cantilevers comparably to anti-CD3–coated cantilevers (Fig. 2 C). However, we found weaker pulling forces (~2-fold less) on pMHC-coated cantilevers compared with anti-CD3–coated cantilevers (Fig. 2 D). We suspect this weaker pulling force was caused by the breaking
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of some noncovalent bonds in the relatively weaker interaction of pMHC with TCR compared with the interaction of anti-CD3 with CD3. Indeed, force spectroscopy and biophysical probe experiments have shown prompt unbinding of individual pMHC–TCR interactions under low pulling forces (Puech et al., 2011; Liu et al., 2014).

We found no correlation between the integrated Ca\textsuperscript{2+} flux and the magnitudes of either the pushing (Fig. 2 E) or pulling (Fig. 2 F) forces. The pushing force by T cells always preceded pulling, resulting in a consistent force–time profile (Fig. 2 A). To identify the temporal correlation between Ca\textsuperscript{2+} rise and the onset of pulling forces (Fig. 2, A and G). The mean (± 95% confidence interval [CI]) of this time lag was 32.5 ± 8.7 s for pMHC and 32.1 ± 7.2 s for anti-CD3. These results show that Ca\textsuperscript{2+} flux and force generation upon triggering are temporally correlated.

We next sought to dissect the molecular mechanisms of this force generation. Actin-rich structures spearhead contact with APCs, as mentioned earlier. Rapid accumulation of F-actin at the immune synapse is a hallmark of T cell activation (Billetteau et al., 2007) and serves important roles in T cell activation (Malissen and Bongrand, 2015). To determine whether actin polymerization was the source of the generated force, we treated cells with Latrunculin A (LatA), which blocks actin polymerization (Kueh et al., 2008). LatA treatment abrogated Ca\textsuperscript{2+} flux in T cells during continuous contact with anti-CD3–coated cantilevers (Fig. 3, A and D). Furthermore, both pushing
(Fig. 3 E) and pulling (Fig. 3 F) forces were absent. These results show that TCR ligation is not sufficient to activate T cells when the actin cytoskeleton is disabled, consistent with published findings (Varma et al., 2006).

In many systems, myosin contractility in conjunction with actin dynamics mediates traction forces. Myosin contractility plays a crucial role in T cell activation by controlling the movement of signaling microclusters at the immune synapse (Ilani et al., 2009; Yi et al., 2012). Myosin IIA activity in T cells is accelerated upon phosphorylation by myosin light chain kinase (MLCK). We tested for the role of myosin in force generation by treating with the MLCK inhibitor ML-7. We observed a significantly diminished pull force upon treatment with ML-7 compared with vehicle-treated cells (Fig. 3, B and F). The mean push force was also decreased, although to a lesser extent (Fig. 3 E). We did not observe differences in the integrated Ca2+ flux with ML-7 (Fig. 3 D). These results suggest that myosin IIA contractility is important in mediating force generation in T cells during activation, especially during the pull phase.

To investigate whether Ca2+ flux regulates the generation of force by T cells, we treated cells with 2-APB, a small-molecule blocker of the calcium release–activated channel (CRAC) (Prakriya and Lewis, 2001; Peinelt et al., 2008). We observed dramatically decreased integrated Ca2+ flux upon treatment (Fig. 3 C), consistent with blockade of Ca2+ entry through CRACs. We observed a significant decrease in the magnitude of the pulling force of 2-APB–treated cells versus controls, but pushing forces were no different (Fig. 3, E and F). This result suggests that pulling requires a sustained, elevated Ca2+ response. We noted, however, that there was a lack of correlation between integrated Ca2+ flux and pulling force (Fig. 2 F). This discrepancy could be reconciled if there were a low threshold of Ca2+ sufficient for achieving a maximal pulling force. We tested this notion by gathering the forces of cells stimulated with anti-CD3–coated cantilevers in the presence of 2-APB and EGTA, both of which had low Ca2+ flux by blocking entry of extracellular Ca2+, as well as vehicle-treated cells, which had a broad range of Ca2+ fluxes. Indeed, we found in fitting the aggregation of these touches that pulling forces were near maximal once the integrated Ca2+ flux was ∼200 (Fig. S2 C).

One explanation for Ca2+-mediated regulation of pulling forces is that Ca2+-dependent calmodulin signaling leads to MLCK activation and, subsequently, pulling forces. Indeed, loss of calmodulin leads to unresponsiveness in T cells (Lin Figure 3. Inhibitor treatments weaken force generation and Ca2+ flux through several mechanisms. Time courses of both force on the cantilever and normalized Fluo-4 intensity. The AFM cantilever was brought into continuous contact with Fluo-4-labeled T cells for 180 s and imaged every 1 s. Flux was normalized to the Fluo-4 intensity at t = 0, when the AFM force trigger was reached upon initial contact. Cells were treated with 1 µM LatA (A), 10 µM ML-7 (B), and 40 µM 2-APB (C). Data were pooled for LatA (n = 14 cells from four independent experiments), ML-7 (n = 10 cells from two independent experiments), and 2-APB (n = 10 cells from two independent experiments). Comparison of integrated calcium flux (D), pushing forces (E), and pulling forces (F) for cells with or without inhibitor treatment. The pooled anti-CD3 results are the same points as in Fig 2. ns, not significant.
et al., 2005). Our experiments perturbing Ca\(^{2+}\) entry showed that at low Ca\(^{2+}\) levels, the magnitude of the pull force became more dependent on integrated Ca\(^{2+}\) concentration. The etiology of this Ca\(^{2+}\) threshold is as yet unknown, and could be caused by calmodulin.

Numerous lines of evidence suggest that T cells “sum up” responses through sequential engagements with antigen on APCs (Faroudi et al., 2003; Henrickson et al., 2008). TCR signaling that was abbreviated induced anergy caused by nuclear factor of activated T cells (NFAT)-driven transcription (Maran-...mittent signal, where single sinusoidal waves were separated by 10 s was insufficient (Pryshchep et al., 2014). To test whether the application of force could rescue signaling in T cells where the actin cytoskeleton was inhibited with LatA, we programmed the piezo z-stage of the AFM to gently engage the T cell with an anti-CD3–coated cantilever, hold position as before for 15 s, then oscillate in a sinusoidal fashion with an amplitude of 500 nm and period of 2 s (Fig. 4 A), akin to the size of invadopodia (Ueda et al., 2011). This sinusoidal movement of the cantilever translated into an ∼180 pN range of forces felt by the cell (Fig. 4 B), on par with the pushing force generated from the cell’s surface (the upward movement of each oscillation) compared with the forces as the cantilever retracted into the surface. Increased engagement of cell receptors over time should result in larger force amplitudes, because the tip would experience increased adhesion forces as the cantilever pulled upwards. Indeed, we saw a significant increase in amplitude when we tested cyclical force in the absence of LatA (Fig. 5, B and C; and Fig. S3 A). Likely, with an intact actin cytoskeleton, the T cell pushed against the oscillating cantilever and formed more contacts with molecules on the tip over time.

In contrast, when we examined the force amplitude in the presence of LatA, we saw little to no rise in the amplitude over time (Fig. 5, A and C). The same was true for cantilevers coated with control mAbs (Fig. S3, C–F; and Fig. 5 C). From these results, we conclude that LatA treatment precluded an
increase in the number of engaged receptors. We therefore posit that cyclic mechanical forces act by increasing the signal per engaged receptor rather than the number of engaged receptors when F-actin is inhibited.

In addition to this work, others have shown that application of external mechanical force on the TCR through anti-CD3 antibodies or pMHC is capable of inducing Ca\(^{2+}\) entry (Kim et al., 2009a; Li et al., 2010). One proposed mechanism for mechanosensation by the TCR is a conformational change model, wherein the mechanical forces are transduced through the TCR–CD3 complex (Kim et al., 2009b), resulting in exposure of buried ITAM motifs (Xu et al., 2008; Das et al., 2015). Oscillating forces delivered exogenously or generated at the lamellipodium (Sims et al., 2007) could be required to unbury these ITAM-bearing domains.

In conclusion, our findings suggest a mechanical–chemical positive feedback loop, whereby initial TCR engagements result in local force generation by the T cell through cytoskeletal rearrangement. These forces enable spreading and rhythmic oscillation of the lamellipodium and act to both increase the number of contacts and enhance signaling from existing ones. Cyclical forces applied to T cells that cannot generate their own forces rescue this process. We showed that application of cyclical force alone with control antibodies elicited negligible Ca\(^{2+}\) responses. Thus, pure mechanical stimulation is not sufficient; rather, the mechanical forces must be delivered through engagement of the TCR. We hypothesize that some short-lived signaling intermediate is formed during force-associated TCR triggering, and the life of that intermediate is less than 10 s, not unlike the same interval observed in previous work (Pryshchep et al., 2014). The identity of this time-dependent signaling pathway is unknown, though our AFM-based approach will be useful for dissecting the role of candidate pathways.

### Materials and methods

#### Mice, cell lines, and reagents

All primary murine CD4\(^{+}\) T cells came from homozygous OT-II TCR transgenic mice (Taconic). The following antibodies were used in this study: biotinylated anti–human-CD25 (clone BC96), biotinylated anti–CD3\(\varepsilon\) (clone 145-2C11), biotinylated anti-CD43 (clone 1B11), and anti-CD28 (clone 37.51) from BioLegend, and anti-CD3\(\varepsilon\) (clone 145-2C11) from Bio X Cell. Biotinylated I-A\(^{a}\) presenting OVA(323–339) was obtained from the National Institutes of Health Tetramer Facility for pMHC studies. Ovalbumin peptide 323–339 was obtained from AnaSpec, and recombinant human interleukin-2 was obtained from Prometheus. Latrunculin A was obtained from Cayman Chemical, ML-7 from Santa Cruz Biotechnology, Inc., and 2-APB from Abcam.

#### Preparation of primary murine CD4\(^{+}\) T cells

T cells from OT-II mice were isolated from spleen and lymph nodes using CD4\(^{+}\) EasySep immunomagnetic separation (STEMCELL Technologies). Cells were activated on plate-bound anti-CD3\(\varepsilon\) (8 µg/ml) with 2 µg/ml anti-CD28 for 2 d before being taken off and maintained in interleukin-2–containing medium. Cells were assayed 4 d after activation.

#### Fluorescence imaging

All fluorescence images were collected on a Nikon Ti-E system fitted with a 40× Plan Fluar objective, NA 0.6. Excitation of fluorophores was done using epifluorescence excitation from a halogen lamp light source (Sutter Instrument). The Chroma 49002-ET-EGFP (FITC/Cy2) filter cube was used for excitation and emission of Flu-4. For pseudoriometric imaging, cells were loaded with 3.75 µM Fura-Red concurrently with 1 µM Flu-4 at 25°C for 45 min. Cells were washed twice before use. To image Flu-4 and Fura-Red intensity simultaneously, we excited the cells using epifluorescence excitation through a 470/40 filter. Emitted light was passed through a Photometrics DV2 Dualview with 525/40 and 630/50 dichroics. The mean intensity of Flu-4 signal was divided by mean intensity of Fura-Red signal to get the ratio shown. Images were collected using an intensified CCD camera (XR/MEGA-10; Stanford Photonics). Image acquisition was controlled using µManager (https://micro-manager.org). Custom code written with MATLAB (MathWorks) using the Image Processing Toolbox was used to identify cell areas for the calcium studies.
Atomic force microscopy
AFM was conducted using an Asylum Research MFP 3D-BIO system combined with a Nikon inverted microscope (Ti-E). The cantilevers used were HYDRA6R-200N (AppNano). Cantilevers were mounted before touching the cell, and inverse optical lever sensitivities and spring constants were calibrated on a bare glass surface. Cells were loaded with appropriate dyes and then allowed to settle on poly-t-lysine–coated 50-μm Fluorodishes (World Precision Instruments). Cells were kept in imaging media (phenol red–free RPMI plus 5% FBS, Hapes, and penicillin/streptomycin). The dish was maintained at 37°C using the Asylum Research Petri heater. In the experiments described, the cantilever was lowered onto cells with the tip positioned approximately at the middle of the cell area. The cantilever tip was gently lowered toward the cell at a speed of 2 μm/s until the specified force trigger was achieved. The cantilever remained in contact with the cell either unmoving or programmed to execute rhythmic piezo-γ movements. Finally, the cantilever was fully retracted. We collected Fluo-4 fluorescence images at 1 frame/s and AFM deflection data at 20,000 Hz. To synchronize acquisition of data from the AFM and microscope, we used custom LabView code and a PCI-6115 board (National Instruments) to acquire the analog deflection and piezo-γ movements. These data were processed to the start and trigger point of each touch, and digital pulses from the AFM controller, digital pulses from the AFM controller corresponding to frames of the camera. These data were processed and analyzed using custom code written in MATLAB.

Chemical conjugation of AFM cantilevers
HYDRA6R-200N tips were plasma-cleaned and bathed in acetone with 4% (3-mercaptopropyl)-trimethoxysilane (Sigma-Aldrich). After 2 h, tips were removed; washed with acetone, isopropanol, and water; and cured for 1 h at 110°C under vacuum.

Streptavidin (Jackson ImmunoResearch Laboratories, Inc.) was reacted with sulfo-LC-SPDP using free amine groups on streptavidin to form covalent bonds. The reaction mix was passed through a Zeba Desalt column (89882; Thermo Fisher Scientific) to remove unreacted cross-linker. The resulting product was then reacted with the free sulphydryl groups of the silanized cantilevers overnight at 4°C. Streptavidin-bonded tips were washed in PBS, bathed in a solution of 25 μg/ml biotinylated antibody (anti-CD3, anti-hCD25, and anti-CD43), and washed again in PBS. For the pMHC-coated tips, streptavidin-bonded cantilevers were bathed in a solution of biotinylated I-Ab-OVA(323–339) at 50 μg/ml.

Statistical analyses
Fluorescence data were scaled to the intensity at the trigger point. Time-integrated fluorescence intensity was calculated using a trapezoidal approximation. Deflection data were decimated down to 20 Hz. Force data were obtained from the deflection in volts by multiplying by inverse optical lever sensitivity and spring constant. The maximum push force was calculated for the largest peak after trigger time. The push force had as its baseline the nearest prior inflection point as determined by custom code in MATLAB, not necessarily the 0-force baseline. Maximum pull force was calculated from the 0-force baseline to the absolute minimum force in the time series.

Permutation testing was used for all statistical comparisons of calcium flux and force generation. Permutation is superior to the classic t test as it does not require a normally distributed population. We used the permutationTest2 function of the Resample package of R (Hesterberg, 2015) to calculate p-values and determine 95% CIs, performing 100,000 permutations. All error boxes in all figures show the bootstrapped mean and 95% CI.

Online supplemental material
Fig. S1 shows the cross-linking strategy, the chemical strategy of covalently attaching streptavidin to the cantilever using a sulfo-LC-SPDP cross-linker. Fig. S2 shows pseudoratiometric imaging of calcium influx, showing that Fluo-4 intensity rise is caused by Ca²⁺ concentration increase, not shape change of the cell. Fig. S3 shows that application of cyclic force in the absence of LatA does not significantly increase signaling strength compared with continuous contact. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201511053/DC1.

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