Structure-activity mapping of the peptide- and force-dependent landscape of T-cell activation

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Abstract

Adaptive immunity relies on T lymphocytes that use αβ T-cell receptors (TCRs) to discriminate amongst peptides presented by MHC molecules (pMHCs). An enhanced ability to screen for pMHCs capable of inducing robust T-cell responses could have broad applications in diagnosing and treating immune diseases. T-cell activation *in vivo* relies on biomechanical forces to trigger activation by sparse antigenic pMHCs. However, *in vitro* screening tests potential pMHCs without force and at high (non-physiological) pMHC densities and thus often fails to predict potent agonists *in vivo*. Here, we present a technology that uses biomechanical force to initiate T-cell triggering in high throughput. BATTLES (Biomechanically-Assisted T-cell Triggering for Large-scale Exogenous-pMHC Screening) displays candidate pMHCs on spectrally encoded ‘smart beads’ capable of applying physiological loads to T cells, facilitating exploration of the force- and sequence-dependent landscape of T-cell responses. BATTLES can be used to explore basic T-cell mechanobiology and T cell-based immunotherapies.

Introduction

The adaptive immune response relies on the ability of T cells to sensitively discriminate non-self from self, allowing mammals to detect pathogen infection or malignant transformations. At a molecular level, this process is mediated by αβ T-cell receptors (TCRs) that recognize rare foreign peptides presented by major histocompatibility complex (pMHC) molecules amongst the ubiquitous self-pMHCs expressed on an antigen presenting cell (APC). TCR recognition of an antigenic pMHC molecule triggers downstream signaling events (*e.g.* Ca\(^{2+}\) flux) that ultimately regulate T-cell activation, with the activation threshold regulated by antigenic pMHC densities on the APC\(^1\). It remains challenging to predict which peptide sequences will engage and activate a particular TCR. Peptide screening methods that simultaneously probe binding and triggering and enhance understanding of TCR-pMHC recognition would have broad applications for immune and infectious disease diagnosis and treatment.

*In vivo*, T cell detection of antigenic peptides is exquisitely sensitive, capable of identifying <10 non-self/altered peptides\(^2,3\) on infected or transformed cells\(^4\), including sequences that differ only by a single amino acid\(^5\). By contrast, measured *in vitro* 3D affinities for known stimulatory peptides are surprisingly weak (K\(_d\) \(\sim\) μM) and often do not differ significantly from non-stimulatory peptides (K\(_d,\)non-self < 10 K\(_d,\)self\(^6\)). Moreover, measured *in vitro* affinities often do not accurately correlate with T-cell activity\(^6-8\): many high-affinity interactions do not stimulate a T-cell response\(^9\), while many potent agonists bind with only weak or moderate affinities\(^10,11\). Together, these paradoxical results establish that equilibrium binding is not sufficient to explain T-cell recognition *in vivo*.

Recent evidence suggests that the pN to nN biomechanical forces generated at TCR-pMHC interfaces during T-cell immunosurveillance and synapse formation may be critical for sensitive and specific recognition\(^12\). Stimulatory pMHCs interacting with TCRs form ‘catch bonds’ whose lifetimes increase with loads up to an optimal force, which fosters robust T-cell activation with only 2 pMHCs per T cell\(^26\). Non-
stimulatory interactions form ‘slip bonds’ whose lifetimes decrease with force\textsuperscript{9, 13, 14}, inducing no response. Screening to identify agonist vs. non-agonist peptides therefore requires an \textit{in vitro} assay capable of quantifying not just binding but downstream activation in the presence of applied loads.

Unfortunately, existing \textit{in vitro} assays are insufficient to address this challenge. Affinity-based screening approaches (\textit{e.g.} pMHC multimer screening\textsuperscript{15-17} and yeast display\textsuperscript{18}) assay $10^{3}$-$10^{9}$ different sequences, facilitating deep exploration of the potential sequence space. However, these assays quantify binding alone (and not activation) in the absence of force and present interactions at artificially high concentrations by using multimers. Functional avidity assays including high-throughput synthetic biology approaches\textsuperscript{19-21} co-culture engineered T cell lines with peptide-pulsed APCs to assess cellular responses, with the potential to recreate force-dependent intercellular molecular interactions resulting from cytoskeletal rearrangements. However, APCs must be pulsed with peptides at high concentrations to elicit T-cell activation, and peptides identified in such high-density screens often fail to drive potent activation under the low-density conditions typical \textit{in vivo}\textsuperscript{1}. By contrast, low-throughput mechanobiology techniques (\textit{e.g.} traction force microscopy\textsuperscript{22-26}, optical trapping\textsuperscript{27, 28}, atomic force microscopy\textsuperscript{29}, biomembrane force probes\textsuperscript{13, 30, 31}, and optomechanical actuator nanoparticle\textsuperscript{32}) exert external well-calibrated shear loads on T cells interacting with specific pMHCs displayed at low densities and can drive robust activation at densities as low as 2 pMHCs/T cell. However, these techniques require extensive costly equipment and are labor intensive, restricting measurements to a limited number of peptide sequences.

Here, we present a technology (BATTLES, for \textbf{Biomechanically-Assisted T-cell Triggering for Large-scale Exogenous-pMHC Screening}) that profiles T-cell signaling responses for thousands of cells interacting with different pMHCs at low densities and in the presence of physiological shear loads. To test and validate BATTLES, we profiled responses of T cell lines (TCR589 and TCR55) previously shown to bind an HIV pol-derived peptide (IPLTEEAEL, HIVpol) with divergent signaling responses. Measurements for >11,000 TCR589 and TCR55 cells interacting with 21 peptides\textsuperscript{9} correctly recapitulated known responses for TCR589 and identified an alternate synthetic peptide sequence (VPLTEDALL) capable of stimulating TCR55 cells with as few as 3 TCR-pMHC interactions at the interface. By applying BATTLES to systematically vary the applied force and density of presented peptides, we further established that TCR55 cells interacting with this alternate synthetic peptide exhibit catch bond-like behavior with the strongest response at 20-30 pN/s loads and the specificities decrease with increasing pMHC densities. Finally, we applied BATTLES to profile responses of a third clinically relevant TCR (DMF5)\textsuperscript{33}. These model systems establish BATTLES as a new technology suitable for obtaining deeper insight into the structure-activity relationships of TCR triggering and discovering novel pMHC antigens under conditions of physiological force.

\textbf{Results}
To apply well-calibrated shear forces, BATTLES deposits T cells onto the surface of hydrogel ‘smart beads’ bearing pMHCs that swell upon small changes in temperature (Fig. 1a). To monitor downstream signaling responses via high-throughput single-cell microscopy, T cells and ‘smart beads’ are loaded into microwell arrays with a Ca\(^{2+}\)-sensitive dye (Fig. 1b). By linking pMHCs to spectrally encoded ‘smart beads’ with a 1:1 relationship between the presented peptide sequence and embedded spectral code, each BATTLES experiment can profile responses of ~1,000-3,000 cells interacting with >20 peptide sequences in <5 hours.

**‘Smart’ beads can apply well-calibrated loads to single cells**

Stimulus-responsive polymers have been used to probe mechanobiology within bulk tissues and for single cells\(^34,35\), including T cells\(^32\). ‘Smart beads’ that shrink and swell can apply homogeneous expansion forces\(^36,37\) (Fig. 1c), with the shear load applied at bead surfaces given by Eqn. 1:

$$F = \frac{2R_{cell}}{\sqrt{\kappa}} \times \frac{\kappa}{R_{bead}} \times \Delta R_{bead}^{1.5}$$

Eqn. 1

where \(R_{cell}\) is the T cell radius (~4 \(\mu\)m), \(\kappa\) is the modulus of rigidity, \(R_{bead}\) is the starting bead radius, and \(\Delta R_{bead}\) is the radius change of the bead. Temperature-responsive polymers such as poly(N-isopropylacrylamide) (PNIPAm) change size at a critical temperature (Fig. 1d) that can be tuned to a physiological range (e.g. 34-35°C) by adding hydrophilic comonomers (e.g. acrylic acid (AAc) or sodium acrylate (SAC))\(^38\). Here, we used a microfluidic droplet generator\(^39\) to produce monodisperse NIPAm droplets containing 55 mM SAC (radius = 16.10 ± 0.4 \(\mu\)m, mean ± SD) (Fig. S1a,b; Movie S1) and then polymerized these droplets into solid beads via batch exposure to UV light, yielding ~ 23.75 \(\mu\)m radius beads (CV = 6.5%) at room temperature (Fig. S1c).

To quantify changes in radii upon cooling, we loaded 12 beads onto an indium tin oxide (ITO) glass slide and imaged while raising the temperature to 37°C for 60 s and then allowing the temperature to cool to 34°C over 120 s (Fig. 1e; Movie S2). ‘Smart bead’ radii shrunk by 1.49 ± 0.16 \(\mu\)m after heating and then expanded by 0.81 ± 0.06 \(\mu\)m after cooling (Fig. 1f,g); after 60 s, the thermo-response reached equilibrium and the bead radius remained constant. A 37°C to 34°C temperature change has only minimal effects on early immune cell activation\(^40,41\), suggesting signaling responses should reflect the response to force alone.

Next, we measured the modulus of rigidity (\(\kappa\)) for a hydrogel slab comprised of identical concentrations of NIPAm monomer and SAC (Fig. S2a). The measured modulus was ~3 kPa (Fig. S2b), similar to rigidities for epithelial cells that support T-cell crawling (4.6 ± 2.2 kPa)\(^42\) and lymph nodes (~4 kPa)\(^43\). Combining measured radii, changes in radii, and moduli yields estimated ramping forces of 20 ± 2 pN/s (Fig. 1h), similar to forces applied by T cells (~21.5 pN/s\(^24\) with forces applied over minutes\(^44\)).
Spectrally encoded ‘smart’ beads allow simultaneous testing of many potential antigenic sequences

Understanding how peptide sequence dictates T-cell signaling requires the ability to systematically vary presented sequences while assessing downstream responses. Spectrally encoded beads allow application of force to multiple TCR-pMHC interactions in a single experiment, and we previously produced poly(ethylene glycol) diacrylate (PEG-DA) hydrogel beads containing >1,000 different spectral barcodes (MRBLES, for Microspheres with Ratiometric Barcode Lanthanide Encoding)\(^{39,45,46}\) (Fig. 2a). To test if PNIPAm is compatible with spectral encoding, we generated carboxylated PNIPAm beads containing a constant amount of Europium (Eu) and 21 combinations of Dysprosium (Dy) and Samarium (Sm) (Fig. 2b, Table S1). Images of pooled beads analyzed with custom software\(^{47}\) revealed 21 distinct clusters of per-bead Sm/Eu and Dy/Eu ratios that were consistent with expected target ratios (Fig. 2c). Cluster variances were similar to those for PEG-DA beads\(^{45}\) (Fig. S3a), suggesting a coding capacity of 48, 80, or 143 codes for clusters separated by 5, 4, or 3 standard deviations, respectively (Fig. S3b,c).

‘Smart’ beads can display peptides at low physiological densities to mimic in vivo conditions

Next, we coupled to these beads a library of HLA-B35 loaded peptides containing systematic mutations within an HIVpol-derived peptide (Pol448–456, IPLTEEAEL); these pMHCs previously bound 2 TCRs (TCR55 and TCR589) in a yeast display screen and drove activation with EC\(_{50}\) values from 1.2 μM to >100 μM\(^{9}\) (Fig. 2d,e, Table S2). To display peptides, we covalently coupled streptavidin (SA) to carboxyl groups, loaded peptides of interest in the B35 MHC groove of biotinylated pMHC complexes via UV-facilitated exchange\(^{48}\), and then incubated biotinylated pMHC complexes with SA-coated beads (Fig. 2f). All UV exchanges and coupling reactions took place in separate volumes to uniquely link peptide sequences with embedded spectral codes. Codes remained easily distinguishable from one another after SA coupling (Fig. S4a); incubation of SA-coated and uncoated beads with ATTO-647-labeled biotin established that SA coupling was uniform and specific (Fig. S4b-d).

Applying shear forces to bead-interacting T cells under physiological conditions further requires that pMHC complexes be conjugated to beads at low densities. To quantify interfacial pMHC density, we incubated beads with fluorescently-labeled anti-β2 monoclonal antibodies (which recognize the β2 microglobulin within peptide-loaded pMHCs) and imaged bead surfaces via total internal reflection fluorescence microscopy (Fig. 2g). Smart beads settled on coverslip surfaces yield an observable spot ~50 μm in diameter (Fig. 2h), with individual antibody-labeled interfacial pMHCs appearing as scattered fluorescent punctae. Over 10 beads, we observed 29 ± 9 pMHCs per ~840 μm\(^2\) bead surface, corresponding to 3-4 pMHCs per 100 μm\(^2\) T cell surface (assuming a 4 μm T cell radius\(^{49}\)) (Fig. S5a); negative control beads displayed only 4.4 ± 1.8 pMHCs per bead surface (Fig. S5b). Interfacial densities were uniform across codes (~0.03 to 0.045 molecules/μm\(^2\), corresponding to 3-4.5 molecules/T cell) (Fig. 2i).

For all 21 codes, mean bead radii and changes in radii upon cooling ranged from 23.0-24.6 μm and 0.78-0.9 μm, respectively (Figs. S6-7), and hydrogel rigidity was fairly constant (κ = 2.48-3.15 kPa) (Fig. S8).
Together, these measurements yield force ramping rates of 20-27.5 pN/s over ~60s (Fig. S9), differing by <27% across codes.

**Pairing T cells with ‘smart’ beads for high-throughput monitoring of force- and sequence-dependent activation**

Early T-cell activation is associated with elevations in intracellular free calcium\(^{50}\), allowing visualization of T cell responses using a calcium-sensitive dye\(^{13, 26, 27, 29-32, 51-53}\). To allow high-throughput imaging of individual T cells upon application of shear force to pMHC-TCR interactions, we fabricated a device containing 1440 microwells (~50 µm in diameter and ~56 µm in depth) that allowed sequential loading of a single ‘smart bead’ within each well followed by 1 or multiple T cells on top (Fig. 3a,b; Movie S3). After an initial incubation to allow cells to interact with and attach to bead-bound pMHCs, we increased the device temperature to 37°C for 1 min followed by cooling to 34°C for 2 mins; subsequent imaging of Ca\(^{2+}\) flux via Cal-520 fluorescence for bead-associated cells over 10 min (4 s intervals) allowed direct monitoring of single T cell signaling. After each experiment, we imaged beads across lanthanide channels to identify the embedded spectral code and thus the displayed peptide sequence (Fig. S10).

**BATTLES correctly identifies peptide agonists known to drive cytotoxic killing in vivo**

To demonstrate BATTLES, we profiled two TCRs (TCR589 and TCR55) previously shown to bind HIVpol pMHCs at µM affinities (\(K_D = 4 \mu M\) and 17 \(\mu M\)) with divergent downstream responses\(^9\). While TCR589 T cells bound HIV-pol tetramers and secreted IL-2 in a dose-dependent manner, TCR55-transduced cells failed to secrete detectable IL-2 despite only ~4x weaker HIVpol binding. If BATTLES can reliably identify stimulatory peptides, we expect to see HIVpol-induced Ca\(^{2+}\) flux for TCR589.

To test this, we quantified Ca\(^{2+}\) flux for 961 TCR589-transduced T cells interacting with all 21 bead-bound pMHCs (Fig. 2c; Table S2). Brightfield images demonstrate loading of ‘smart beads’ within all microwells (Fig. 3b); movies acquired during device heating and cooling establish that cooling swelled beads as expected and that T cells remained in contact with bead surfaces throughout (Fig. 3b; Movie S4). After cooling, fluorescence images of force-induced Ca\(^{2+}\) flux across all peptides (66 ± 18 beads and 46 ± 15 cells per peptide, Fig. S11) revealed a variety of behaviors (Fig. 3c-e). While some T cells exhibited substantial and rapid increases in intracellular calcium (typical of a type-α calcium response indicating successful triggering) (Fig. 3d, top), most cells showed decreasing fluorescence intensities over time, reflecting an absence of triggering (type-β calcium response) combined with photobleaching (Fig. 3d, bottom; Fig. S12a). This is consistent with prior literature suggesting that only a fraction of cells are activated at low pMHC densities, even under optimal force\(^{51}\).

To probe for sequence-dependent differences in type-α calcium responses, we integrated per-cell fluorescence over time and then analyzed only ‘positive’ cells that increased in fluorescence (Eqn. 2):
For TCR589 cells, the HIVpol peptide (IPLTEEAEL) yielded high percentage of ‘positive’ T cell responses (Fig. S12b; Movie S5) and ‘positive’ cells had the largest integrated intensity signals (~2-fold greater than for other peptides (Figs. 3e-g, S13; bootstrapped p = 10^-4, see Methods). Results were consistent across technical replicates (Figs. 3h, S14-15; r^2 = 0.41), establishing that BATTLES can identify peptide epitopes capable of producing a robust cytotoxic response in vivo.

Applying BATTLES to identify novel peptide agonists

Next, we tested if BATTLES could identify alternate potent agonists for TCR55-tranduced T cells that bind HIVpol without activation (Figs. 4a, S16; 67 ± 24 beads and 51 ± 23 cells per peptide). In contrast with TCR589, the HIVpol peptide IPLTEEAEL did not induce significant Ca^{2+} responses; instead, a mutant peptide (VPLTEDALL) produced the strongest functional response across 2 biological replicates in which we shuffled the identity of the peptide associated with each spectral code (Figs. 4b-d, S17-19; bootstrapped p = 10^-5 and 8×10^-4 for each replicate; r^2 = 0.62 between replicates).

To confirm that observed Ca^{2+} responses required force and were not simply due to changing temperatures, we repeated the TCR55 assay after heating beads to either 34 °C or 37 °C without subsequent cooling (Fig. S20a,b). In both cases, integrated Ca^{2+} signals across tested peptides were 2-fold lower and a lower percentage of cells yielded positive integrated Ca^{2+} signals (Fig. S20c); in addition, no peptide produced a response significantly higher than the others. Although some shrinking force may be applied during heating to 37°C, the directions of the applied force and cell contractions (~6 nN after attachment^24) are aligned at interaction surfaces such that the net applied force is negligible.

Multiplexing applied loads can elucidate ‘catch’ vs ‘slip’ bond behavior in a single experiment

For ‘catch’ bonds (found in TCR-agonist pMHC interactions), bond lifetimes increase with force up to an optimal load (typically 8-20 pN) and then decrease; for ‘slip’ bonds (found in TCR-self pMHC interactions), bond lifetimes monotonically decrease with load^9,13,14 (Fig. 5a). If the VPLTEDALL peptide serves as a potent agonist for TCR55-transduced T cells, we expect to see the strongest triggering at an optimal force, with weaker responses at higher or lower forces. To test this, we leveraged ‘smart beads’ to simultaneously vary the peptide sequence and force applied within a single experiment.

Incorporating different amounts of SAc within the ‘smart bead’ matrix tunes the magnitude of the applied force by altering the relative hydrophilicity/hydrophobicity ratio and thus the change in radius upon temperature-induced swelling (Fig. 5b). To apply a range of forces, we polymerized ‘smart beads’ containing 50 mM, 55 mM, or 60 mM SAc, respectively (Fig. 5b). After polymerization, bead radii were constant across all 3 formulations (24.12 ± 1.45 µm, 23.68 ± 1.55 µm, and 24.08 ± 1.66 µm, mean ± SD)
but the change in radius upon cooling from 37°C to 34°C varied substantially (1.44 µm (34 beads, CV = 1.5%), 0.96 µm (24 beads, CV = 5.4%), and 0.64 µm (23 beads, CV = 2.5%)) (Figs. 5b-c, S21; Movie S6). As the modulus of rigidity is unchanged by small changes in SAc concentration, these variations yielded ramping forces of 54.43 ± 1.52 pN/s, 26.96 ± 2.37 pN/s, and 15.57 ± 0.62 pN/s (Figs. 5d, S22).

Prior work using a BFP-based bond lifetime assay established that for TCR55, VPLTEDAEL and VPITEDSQL peptides exhibit maximum lifetimes at 14 pN and 7 pN, respectively, indicating the formation of a ‘catch’ bond, while the HIVpol peptide (IPLTEEAEL) shows ‘slip’ bond behavior (Fig. S23). Here, we profiled the force-dependent responses for these 3 peptides and the VPLTEDALL peptide that drove the largest integrated Ca2+ signal in BATTLES (Fig. 4b-d) via an assay in which each spectral code corresponded to a particular sequence/force combination (4 peptide sequences × 3 forces/sequence = 12 codes) (Fig. S24; Table S4). For VPLTEDALL, measured Ca2+ flux increased with low and moderate forces and then decreased at high force, validating that BATTLES can accurately identify peptide agonists capable of forming ‘catch’ bonds (Fig. 5e). VPITEDSQL triggered the strongest response at low force, consistent with its previously measured catch bond profile; VPLTEDAEL responses were uniformly low across all forces and HIVpol (IPLTEEAEL) showed no significant difference between tested forces compared to no force (p=0.06 via bootstrapping) (Fig. 5e). Results were consistent across a biological replicate in which embedded codes and peptide sequences were shuffled (Table S4; Figs. S25-26; r² = 0.80 across replicates).

**Multiplexing pMHC sequences and concentrations to reveal dose-dependent immunogenicity**

These results suggest that VPLTEDALL triggers the strongest Ca2+ signaling response for TCR55-transduced T cells, at odds with prior peptide-pulsed APC co-culture results identifying VPLTEDAEL as the most potent agonist (Table S3). One explanation for this discrepancy could be the difference in presented pMHC concentrations: peptide-pulsed APCs present peptides at densities > 100 nM (corresponding to ~20 or 300 pMHCs/T cell at 100 nM and 10 µM, respectively54), while BATTLES ‘smart beads’ present pMHCs at physiological densities of 3-4.5 pMHCs/cell. To test if signaling responses depend on presented densities, we repeated BATTLES assays with the same 4 TCR55-binding peptide sequences (VPLTEDALL, VPITEDSQL, VPLTEDAEL, and IPLTEEAEL) at 3 effective concentrations (1X (3-4.5 pMHCs/T cell); 7X (21-31.5 pMHCs/T cell); and 27X (81-121.5 pMHCs/T cell)) (4 sequences x 3 concentrations = 12 codes) (Figs. 5f, S27-28; Table S5). At 1X concentrations, VPLTEDALL again drove the strongest downstream response (Figs. 5g-i, S29-30; bootstrapped p = 0.0075; r² = 0.64 between replicates). For 7-fold higher pMHC densities, the integrated Ca2+ signal for VPLTEDALL remained constant but the signals associated with the other 3 peptides increased and showed no significant difference across peptides, indicating a loss of specificity (Figs. 5g-i, S29-30). At 27-fold higher pMHC densities, the integrated Ca2+ signal associated with the VPLTEDAEL increased even further to drive the strongest response (Figs. 5g-i, S29-30; bootstrapped p = 0.012 for two replicates), consistent with prior observations from tetramer screening and co-culture assays. These results underscore the importance of screening at physiological pMHC densities for accurate identification of likely potent agonists in vivo.
Finally, we applied BATTLES to profile responses for a third TCR with a known potential therapeutic role. DMF5 recognizes the MART-1 melanoma antigen presented by the class I MHC protein HLA-A*0201, and clinical trials of DMF5-transduced T cells interacting with MART-1_{26-35} peptide-loaded dendritic cells demonstrated robust tumor regression. Prior yeast display experiments revealed two distinct classes of peptides that bound the DMF5 TCR: (1) MART-1-like peptides containing a hydrophobic core sequence (GIG in P4-6) and (2) peptides instead containing a highly charged central core (DRG in P4-6), with DRG class peptides binding in a unique 'shifted register' conformation. Tetramers of the MART-1 anchor-modified dodecamer (ELAGIGILTV) showed the strongest binding and the strongest downstream response in peptide-pulsed APC co-culture assays while IMEDVGWLNV was the most potent DRG class peptide; however, it remained unclear whether both peptide classes would drive similar levels of T cell activation when presented at low densities.

To assess reactivities under physiological densities, we applied BATTLES to DMF5 T cells interacting with 11 peptides: 5 GIG class peptides (including the MART-1 anchor-modified dodecamer), 5 DRG class peptides, and a negative control peptide (Tax, LLFGYPVYV) (124 ± 34 beads and 75 ± 29 cells per peptide). Consistent with its known biological role, the MART-1 anchor-modified dodecamer (ELAGIGILTV) drove by far the strongest Ca^{2+} response across two replicates (Figs. 6b-d, S32-34; p=10^{-7} and 3×10^{-4} from bootstrapping comparisons with Tax; r^2 = 0.97). From the DRG class, the MMWDRGLGMM (and not the previously identified IMEDVGWLNV) induced the strongest Ca^{2+} flux (bootstrapped p= 10^{-6} and p = 6.5×10^{-3}). Thus, BATTLES can reveal potential cross-reactivity of therapeutical TCRs towards different epitopes, even for motifs sharing no significant similarities, providing clinically relevant information predicting off-target effects.

**Discussion**

T cells exert shear forces during immunosurveillance and immune synapse formation on an APC. Such physical loads on the TCR-pMHC bond can enhance sensitivity and specificity of recognition by facilitating discrimination between ‘catch’ and ‘slip’ bond-forming complexes. Recapitulating sensitive T cell activation in vitro therefore requires the ability to: (1) display a peptide capable of forming a catch bond with a given TCR at low (physiological) densities, (2) apply shear forces to drive mechanotransduction, and (3) monitor downstream T signaling. While a wide variety of mechanobiology tools have been developed to identify potent peptide agonists, the BATTLES platform is the first (to our knowledge) that recapitulates these physicochemical cues and screens many peptide sequences for their potential to not just bind TCRs but also activate downstream signaling responses.

The TCR-transduced T cell lines employed in typical in vitro co-culture assays are significantly less sensitive than primary T cells, requiring APC pulsing with high peptide concentrations (~ 1 µM) to drive
activation. However, several lines of evidence suggest that peptides identified in such screens may not represent the most promising antigen vaccine candidates. The identity of synthetic peptide sequences capable of stimulating IL-2 cytokine release and CD69 expression for crawling primary T cell blasts\textsuperscript{59} displaying 2B4 and 5cc7 TCRs\textsuperscript{18} depends on the concentration at which they are displayed. It has been shown that the viral antigen density also regulates the reactivity pattern of CD4 T-cell responses \textit{in vivo}\textsuperscript{1}. These results are consistent with the concentration multiplexing BATTLES results presented here. The ability to accurately identify and discriminate potent agonists presented at low pMHC concentrations even when using TCR-transduced T cell lines suggests that BATTLES could be a bona fide proxy to screening primary T cells.

Accurately mimicking physiological conditions requires matching not just the magnitude of applied forces but also the ramp rate and overall duration over which forces are applied. While the estimated force ramp applied by BATTLES ‘smart beads’ (20-30 pN/s) is similar to that applied during traction force microscopy\textsuperscript{24}, optical trapping\textsuperscript{27}, and atomic force microscopy\textsuperscript{30}, critical differences in how forces are applied may allow BATTLES to more accurately mimic \textit{in vivo} physiology. First, while polystyrene or silica beads used in optical trapping assays distribute applied forces across all formed pMHC/TCR interactions at the interface, ‘smart beads’ apply force locally via pMHCs attached to PNIPAm filaments within the bead hydrogel matrix. This geometry, analogous to interactions between APC-presented pMHCs and protrusive T-cell microvilli prior to activation\textsuperscript{60}, may more accurately mimic TCR/pMHC interactions \textit{in vivo}. Second, the duration of the force ramp applied by ‘smart beads’ over 60 s of cooling and associated swelling may allow peptides with a fast 2D on-rate to iteratively rupture and rebind, driving dynamic\textsuperscript{61} and reversible \textasciitilde10 nm structural transitions within the TCR/pMHC complex\textsuperscript{62}. Such energized work (10 nm transitions under 10-15 pN loads) may lead to a stronger and more sustainable Ca\textsuperscript{2+} flux\textsuperscript{27} similar to that observed for naïve T cells\textsuperscript{63}.

In future work, BATTLES can be applied to a wide range of additional questions. While here we assay only 21 peptides, lanthanide-based spectral encoding has previously yielded >1,100 codes\textsuperscript{46}, suggesting BATTLES can be applied to much larger libraries in the future. Expanded peptide libraries could be used to systematically profile how variations in TCR and presented peptide sequences alter activation, providing new insights into the biophysical mechanisms underlying ‘catch bond’ formation. In medicine, applying BATTLES to screen affinity-matured TCR or chimeric antigen receptor (CAR)\textsuperscript{64} T cells for cross-reactivity prior to autologous transfer could reduce the risk of off-target toxicity. In its current form, BATTLES requires <5 hours from preparation of pMHC-coated beads to Ca\textsuperscript{2+} flux imaging and requires \textasciitilde20,000 ‘smart’ beads and \textasciitilde1.2 million T cells for 3 devices. By contrast, coculture assays require \textasciitilde2-4 million each of APCs and T cells and require \textasciitilde18 hours to detect expression of CD69/CD137 or release of cytokines and extra hours for downstream flow-cytometry or enzyme-linked immunosorbent assays. These reduced material and time requirements could pave the way for direct testing of patient-derived T cell samples to identify candidate vaccine neoantigens capable of driving robust activation of
endogenous T cells. Finally, BATTLES could be applied as a general mechanobiology tool for other immune cells, adhesion cells, and even neurons\textsuperscript{65}.

**Methods**

**Production of lentivirus**

LentiX cells were seeded in 6-well plates with 0.6 x10\textsuperscript{5} cells per well in 2 mL complete DMEM (ThermoFisher). The day after seeding, for each well, 750 ng lentiviral vector, 500 ng psPAX and 260 ng pMD2G were mixed with 4.5 mL Fugene (Promega) in 100 mL Opti-MEM and incubated at room temperature for 20 minutes. After incubation, this DNA/Fugene mixture was added to the LentiX cells. The lentivirus supernatant was collected 2 or 3 days after the transfection and filtered to remove dead cells.

**Cell line generation**

Lentivirus encoding transfection of TCR\(\alpha\) and TCR\(\beta\) chain genes was generated as described above\textsuperscript{1}. Briefly, we cloned either the full-length TCR\(\alpha\) or TCR\(\beta\) chain gene into the pHR lentiviral vector. After lentivirus production, 2 mL of the supernatant containing TCR \(\alpha\) and \(\beta\) chains with 1:1 expression was used to infect 1 million SKW3 cells. Following infection, we added 1.5 mL fresh completed RPMI to the mixture. After 2 days, we selected for SKW3 cells with surface-expressed TCR\(\alpha\)\(\beta\) using anti-TCR (Biolegend clone IP26) staining and flow cytometry (Sony SH800 sorter).

**Cell culture**

SKW3 cells transduced with TCR clones 55, 589 and DMF5 were cultured in RPMI 1640 GlutaMAX (Thermo Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich), 100 U/ml penicillin, and 100 U/ml streptomycin (Life technology). Prior to performing all experiments, we confirmed that cells had reached the log phase of growth (1.5 million to 2 million cells/mL), as cells in the lag phase may generate insufficient Ca\(^{2+}\) flux (https://osf.io/xs7zf/).

**Microfluidic device design**

Molding masters for both microfluidic devices used in the paper (the parallel flow focuser for high-throughput generation of the spectrally encoded ‘smart beads’ and the microwell array for on-chip loading of beads and T cells) were designed in AutoCAD (Autodesk). The microfluidic droplet generator was largely similar to our previously published design\textsuperscript{2} except that the width of orifice channels and all channel heights were changed to 25 \(\mu\)m to yield polymerized beads \(\approx\)24 \(\mu\)m in diameter. All transparency masks used for fabrication of molding masters were printed at 50K dpi (Fineline Imaging). Designs of all devices are provided as Supplemental Files and in an associated OSF repository (https://osf.io/xs7zf/).

**Microfluidic molding master fabrication**
‘Smart bead’ generator

We fabricated the molding master for the parallel flow focuser device with a target height of 25 μm by:

(1) Spin-coating SU-8 2025 negative photoresist (MicroChem) (500 rpm for 10 s with acceleration of 133 rpm/s; 3500 rpm for 30 s with acceleration of 266 rpm/s) on a 4-inch test-grade silicon wafer (University Wafer, South Boston, MA);

(2) Soft baking the coated wafer (1 min at 65°C; 5 min at 95°C; 1 min at 65°C);

(3) Exposing the baked wafer using a UV mask aligner (Karl Suss MA6) for 26.8 s at 5.6 mW/cm²;

(4) Post-exposure baking (1 min at 65°C; 5 min at 95°C; 1 min at 65°C);

(5) Developing using SU-8 developer (Microchem Corp, Newton, MA) for ~4 min.

Microwell device for pairing beads and T cells

For the microwell device, we fabricated the microwell layer and roof layer separately on two 4-inch test-grade silicon wafers. Microwell features were fabricated using SU-8 2050 with a target height of 56 μm by:

(1) Spin-coating SU-8 2050 (MicroChem) (500 rpm for 10 s with acceleration of 133 rpm/s; 2750 rpm for 30 s with acceleration of 266 rpm/s) on a 4-inch test-grade silicon wafer;

(2) Soft baking the coated wafer (2 min at 65°C; 7 min at 95°C; 2 min at 65°C);

(3) Exposing the baked wafer using a UV mask aligner for 31.7 s at 5.6 mW/cm²;

(4) Post-exposure baking (2 min at 65°C; 6 min at 95°C; 2 min at 65°C);

(5) Developing using SU-8 developer for ~6 min.

The roof features were fabricated on another wafer using SU-8 2050 with a target height of 100 μm by:

(1) Spin-coating SU-8 2050 (500 rpm for 10 s with acceleration of 133 rpm/s; 1650 rpm for 30 s with acceleration of 266 rpm/s) on a 4-inch test-grade silicon wafer;

(2) Soft baking the coated wafer (5 min at 65°C; 16 min at 95°C; 5 min at 65°C);

(3) Exposing the baked wafer using a UV mask aligner for 41.1 s at 5.6 mW/cm²;

(4) Post-exposure baking (4 min at 65°C; 9 min at 95°C; 4 min at 65°C);

(5) Developing using SU-8 developer for ~9 min.
Silane vapor deposition

To prevent sticking of PDMS during device fabrication, we treated all wafers with trichloro(1H,1H,2H,2H-perfluorooctylsilane) (PFOS, Sigma-Aldrich) by placing wafers in a vacuum chamber along with an uncapped bottle of PDMS with a few PFOS droplet in the cap, pulling vacuum for 1 min, and then maintaining vacuum (without active vacuum) for an additional 9 minutes.

Microfluidic molding master fabrication

All microfluidic devices were fabricated using standard soft lithography protocols.\(^3\)

'Smart bead' generator

Molding masters were used to cast single-layer parallel flow-focuser devices composed of a 1:5 ratio of poly(dimethylsiloxane) crosslinker: base (PDMS, RTV 615, R.S. Hughes). After baking, devices were assembled using the 'jumper cable' strategy described previously.\(^2\)

Microwell pairing device

The microwell layer of the pairing device was cast using a 1:5 ratio of PDMS crosslinker (20 g crosslinker, 100 g base). After mixing in a THINKY mixer (3 min mixing followed by 3 min degassing), the PDMS mixture was poured on top of the microwell mold and this mold/PDMS assembly was placed in a vacuum chamber and subjected to vacuum for ~45 minutes to remove any air bubbles trapped inside the features. After vacuum, the PDMS-coated wafer was spun for 2 min at 200 rpm with 133 rpm/s acceleration using a spin coater (Laurell WS-650) to yield a final PDMS slab height of ~400 μm. The mold with PDMS was then baked at 80°C for 25 min in a convection oven to partially polymerize this layer. Then microwell layer was then peeled off from the mold, flipped over, and adhered to a cover glass (48×65 mm, GOLD SEAL No. 1). The roof layer was casted with a 1:10 ratio of PDMS crosslinker (10 g crosslinker, 100 g base), subjected to vacuum for 45 min, and then baked at 80°C for 30 min. The roof layer was then peeled from the wafer, inlet holes were punched using a catheter punch (SYNEO, 0.025" ID × 0.035" OD, Part No: CR0350255N20R4) to fit the outer diameters of PEEK tubing (ZEUS, 0.010" ID × 0.020" OD) and steel blunt pins (New England Small Tube, Part No: NE-1310-02), and aligned on top of the microwell layer. This two-layer device assembly was then baked at 80°C for 14 hours to fully polymerize and bond both layers. Longer baking time is not recommended as that may enhance the hydrophobicity and shrink the microwells.

Production of lanthanide-encoded ‘smart beads’

Aqueous lanthanide mixtures

For ‘smart bead’ synthesis, N-isopropylacrylamide (NIPAM), poly(ethylene glycol) diacrylate (PEG-DA, Mn=700), sodium acrylate (SAc), acrylic acid (AAc), lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) were purchased from Sigma-Aldrich and used directly without further purification. SAc solution
(pH=7, c[Ac]=1 M) was obtained by adding 5M NaOH into 1M AAc solution until the pH reached 7.0. The poly-acrylic acid wrapped lanthanide nanophosphors (Lns) were synthesized as described previously. Pre-mixed Ln/polymer mixtures containing NIPAM monomer, Lns, PEG-DA, SAc and LAP were generated by varying ratios of three monomer master mixtures each containing different Lns (Table S1). The “Eu”, “Dy” and “Sm” master mixtures also contained 16.3% v/v YVO4:Tm (50 mg/mL), 16.3% v/v YVO4:Dy (50 mg/mL), 16.3% v/v YVO4:Sm (50 mg/mL), respectively. A total amount of 21.3% v/v Lns maintain an equal amount of hydrophilic content across all the formulas. All master aqueous mixtures in Table S1 contained purified water with 9.2% w/v NIPAM and 5% v/v YVO4:Eu (50 mg/mL), 2.8437% v/v PEG-DA, and 5.5% v/v SAc solution. For the force multiplex experiment, 5% v/v and 6% v/v SAc solution were added into the master aqueous mixture for high and low force ramps, respectively.

**Droplet generation**

2.5% v/v LAP (39.2 mg/mL in DI water) was added to each solution right before its injection into the droplet generator. Pre-mixed Ln/polymer mixtures and HFE7500 with Ionic Krytox (IK) and 0.05% v/v AAc were flowed into the aqueous inlet and oil inlets of the droplet generator device, respectively, yielding high-throughput production of pre-gel droplets at the flow-focusing nozzle. Droplets (radius=16.1 ± 0.4 μm) were generated with aqueous and oil flow rates of 500 μL/h and 3200 μL/h and then collected through Tygon tubing into a 24-well plate (Thermo Fisher Scientific). ~80 μL running oil was added into the each well prior to the collection of the droplets to prevent evaporation of HFE7500 and resultant droplet breakage.

**Bead polymerization and functionalization**

During droplet generation, the AAc in the oil phase gradually diffuses into the aqueous phase to form a carboxy shell to allow subsequent covalent coupling of streptavidin to the bead surface. For 2 formulas at a time, we applied flood UV light (IntelliRay, UV0338) at 100% amplitude (7” away from the lamp, power= ~50-60 mW/cm²) for 2 minutes to induce polymerization and crosslinking of carboxyl groups at interfacial surfaces. After polymerization, beads were transferred into a 2 mL fritz column with ~20 μm pore size (Biotage) and then washed with: 2 mL dimethylformamide (DMF, Thermo Fisher Scientific) for 20 s; 2 mL dichloromethane (DCM, Thermo Fisher Scientific) for 10 s; and 2 mL methanol (Thermo Fisher Scientific) for 20 s. After washing, beads were resuspended in 1 mL PBST buffer for the aqueous EDC chemistry.

**EDC chemistry for streptavidin surface conjugation**

To functionalize ‘smart’ beads with streptavidin, 150 μL of carboxy ‘smart’ beads (~200,000 beads) were washed with 200 μL 0.1 M MES buffer (pH = 4.5) supplemented with 0.01% (v/v) Tween-20 (activating buffer) three times prior to resuspension in 200 μL of activating buffer. Next, 200 μL of a freshly made 2%w/v 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma-Aldrich) in activating buffer was added to the bead solution. The entire reaction was then incubated for 3.5 hours at room temperature on a rotator with end-over-end mixing (10 rpm). The bead slurry was then washed with 1 mL 0.1 M borate
buffer (pH = 8.5) supplemented with 0.01% (v/v) Tween-20 (conjugating buffer) and subsequently resuspended in 400 μL of conjugating buffer. Conjugation of streptavidin (Sigma-Aldrich) was carried out by adding 16 μL streptavidin solution (dissolved in 1X PBS at 1 mg/mL) into the mixture and rotated the whole slurry overnight at 4°C. The reaction was quenched by adding 10 μL of 0.25 M ethanolamine in conjugating buffer to the mixture and rotating for 30 minutes at 4°C. The final product was washed 3 times with PBST buffer and resuspended in 200 μL of the same buffer for subsequent bright field and fluorescence imaging. The efficiency and consistency of streptavidin coupling was checked by incubating 0.5 μL 1 mg/mL Biotin-Atto647 and ~5 μL bead slurry (~500 beads, either streptavidin-functionalized or without streptavidin as a negative control) for 1 h at room temperature and imaging in the Cy5 channel.

Production of inclusion bodies of B35 MHC heavy chain and human b-2-microglobulin

The codon-optimized B35 MHC heavy chain gene or human β-2-microglobulin gene was cloned into a pET28a vector. The construct was transformed into BL21 (DE3) E. coli and grown over night on LB agar plates supplemented with kanamycin. Single colonies were then picked, used to inoculate 10 mL LB broth containing kanamycin, and then grown overnight at 37°C with shaking at 250 rpm. The 10 mL overnight culture was added into 1 L of LB broth containing kanamycin and grown until OD= 0.5. IPTG was added into the same culture at a final concentration of 1 mM and shaken for another 3 hours. The bacteria were pelleted down by centrifuge at 6,000g for 30 min and then resuspended in 50 mL Buffer 1 (50 mM Tris-HCl, pH=8, 100 mM NaCl, 1mM DTT, 5% Triton-X-100, 1 mM EDTA). The bacteria were then sonicated with 2-second sonication and 2-second rest for 2 min and rested for another 2 min on ice. The sonication was repeated for 4 times. The lysed bacteria were then pelleted down at 6,000 g for 15 min, washed in 50 mL Buffer 1, and subjected to the same sonication procedure two more times. The pellet was then washed in 50 mL Buffer 2 (50 mM Tris-HCl, pH=8, 100 mM NaCl, 1 mM EDTA) and subjected to the same sonication procedure 2 more times. Finally, the pellet was solubilized in 25 mL Buffer 3 (8 M urea, 50 mM Tris-HCl pH=8, 10 mM EDTA, 10 mM DTT) by rotating overnight. The inclusion body was run on SDS-PAGE to check the protein molecular weight.

Refolding of peptide-MHC

We prepared 1 L refolding buffer containing 5 M urea, 400 mM L-Arginine, 100 mM Tris-HCl (pH=8), 2 mM EDTA, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione. We then dissolved 30 mg UV-cleavable peptide in 1 mL DMSO and added it into the refolding buffer. Next, we mixed 30 mg B35 MHC heavy chain inclusion body and 30 mg human β-2-microglobulin inclusion body and added this into refolding buffer through a 23G needle by gravity. The refolding buffer was then transferred to dialysis tubing and dialyzed against 10 L 10 mM Tris-HCl (pH=8). Every 12 hours, the 10 mM Tris-HCl (pH=8) buffer was changed to fresh buffer; the dialysis was performed for a total of 60 hours. After dialysis, the refolding buffer was purified by flowing through diethylaminoethyl (DEAE-) cellulose which was equilibrated using 10 mM Tris-HCl (pH=8). The refolded pMHC was eluted from the DEAE-cellulose using 0.5 mM NaCl in 10 mM Tris-HCl (pH=8), buffer exchanged to 10 mM Tris-HCl (pH=8), and concentrated by Amicon concentrator. The protein was then biotinylated overnight at 4 °C. The biotinylated pMHC protein
was then purified via size-exclusion chromatography (GE Superdex 200 increase 10/300 GL) and fractions were analyzed by running on SDS-PAGE. Fractions with refolded pMHC protein were further purified by running on a Mono-Q column. Fractions were again analyzed by SDS-PAGE and fractions with refolded pMHC protein were then flash frozen in liquid nitrogen and stored in -80 °C.

Peptide production

21 peptide ligands (length = 9 aa) were produced by Fmoc-based solid-phase peptide synthesis (GenScript). The UV-labile peptide used here was KPIVVLJGY, where "J" is Fmoc-(S)-3-Amino-3-(2-Nitro-Phenly)-Propionic Acid (also produced by GenScript). All peptides were dissolved in dimethyl sulfoxide (20 mM) and stored at -20 °C until further use.

Peptide exchange reaction

UV-facilitated peptide exchange was performed as described previously 5. Briefly, we first prepared peptide exchange buffer containing 20 mM Tris-HCl (pH 7.0) and 150 mM NaCl. Next, for each exchange reaction, we combined 40.4 μL exchange buffer, 5.6 μL of the peptide to exchange (500 μM, diluted from 20 mM stocks in DMSO in exchange buffer) and 10 μL UV-labile pMHC (2.8 μM in PBST) in one well of a 96-well plate. The plate was placed on a thermo-mixer whose temperature was pre-equilibrated to 4°C. A UVP compact and handheld UV Lamp (365 nm, UVP95000505, 6W) was placed over the sample for 2 h, with the distance between the lamp and sample set to ~3 cm. After that, the sample was withdrawn from the wells and purified and concentrated using 3K spin columns (ThermoFisher) three times at 4°C. The final concentration was estimated using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

pMHC-functionalized ‘smart’ bead

The streptavidin (SA)-coupled ‘smart’ beads were washed with PBST buffer three times. To create sample beads with pMHC at physiological density (for normal BATTLES and force multiplex experiments), we mixed 0.5 μL of 10 nM pMHCs with ~20,000 streptavidin ‘smart’ beads in 50 μL PBST buffer. The mixture was gently rotated for 1 h at room temperature, washed with 200 μL PBST four times, and then resuspended in 200 μL PBST buffer. Bead surfaces were blocked by adding 5 μL of 20 mg/mL biotin-BSA (Thermo Scientific) to the pMHC coated bead slurry for 1 h at room temperature, washed three times in PBST, and then resuspended in 50 μL PBST for further use. The BSA-coated surface eliminates potential non-specific binding of the bead to the T cell 6. For the concentration multiplex experiment, we mixed 0.5 μL of 100 nM (7X) or 500 nM (27 X) pMHCs with ~20,000 streptavidin ‘smart’ beads in 50 μL PBST buffer and followed identical bead-coating procedures. For quantifying the concentration difference, we added 1 μL of alexa-647 labeled anti-beta-2 microglobulin monoclonal antibody (Thermo Fisher, clone: B2M-01, 1 mg/mL) to 5 μL beads solution containing ~2,000 pMHC-coated beads and gently rotated for 1 h. We then washed beads with PBST five times, resuspended in 20 μL 1X PBS buffer, and imaged on an inverted microscope (Nikon Eclipse Ti, Nikon) with a SOLA light engine (380 nm -680 nm, Lumencor, Beaverton, OR) and a Cy5 filter cube set.
pMHC quantification by single molecule TIRF microscopy

To determine the actual density of pMHC molecules on ‘smart bead’ surfaces, we imaged beads via single-molecule total internal reflection fluorescence (smTIRF) microscopy (evanescent wave penetration depth of ~400 nm and a ~50 µm× 50 µm field of view). To detect bead-bound pMHCs, we added 1 µL of PE anti-human β2-microglobulin antibody (BioLegend, clone: A17082A, 0.2 mg/ml) to 5 µL beads solution containing either ~2,000 pMHC- or 2,000 SA-coated beads (for control experiment) and gently rotated for 1 h. After staining, we washed beads with PBST five times, resuspended in 50 µL 1X PBS buffer, and placed samples on ice prior to imaging. Stained beads were imaged by smTIRF microscopy using a simple flow cell created using Scotch double-sided tape, a cover glass (VMR, 22 × 50 mm, #1.5), and a glass slide (Corning, 75 × 25 mm). After loading ~200 beads into the flow cell, the channel was washed with 1X PBS five times and then sealed with nail polish to prevent evaporation during the imaging. For one bead in the field of view, we recorded 10 sequential images at 100 ms intervals. To quantify individual pHMC molecules, we counted the number of bright spots using “Find maxima” in ImageJ with an intensity prominence of 40 and spots with different coordinates were summed across all 10 images.

Shear modulus measurement

To access the modulus of rigidity/shear modulus of each code, we made NIPAm slabs containing identical ingredients as the 21 codes and then added 50 µL of each code to the ~15 mm diameter wells in the lid of 24-well plate. After 2-min UV polymerization, we washed the slab three times and rehydrated in colorless RPMI. We then measured the shear modulus (modulus of rigidity) using the TA rheometer (HR 10, Discovery Hybrid Rheometer) with a 20 mm parallel plate associated with Peltier plate Steel plus Solvent Well. The strain and angular frequency were set to 0.015% and 0.1 rad/s to 100 rad/s with 5 points per decade, respectively. The shear modulus is the root sum square of the storage modulus and loss modulus.

Quantifying bead shrinking and swelling with changes in temperature

All bead imaging used a small dissection scope (AmScope) equipped with a 20X objective with either a 0.5X relay lens or a 1X relay lens (for the force multiplexing beads) and a Thorlabs camera (DCC3240M). Prior to imaging, we washed SA-coated ‘smart’ beads with PBST three times, resuspended in colorless RPMI buffer, and then loaded them into a flow cell. After bead loading, we sealed the flow cell with nail polish (to prevent evaporation) and placed the flow cell on an indium tin oxide (ITO) glass slide (Bioscience Tools, TC-1-100s). While imaging continuously, we: (1) heated the sample from room temperature (25°C) to 37°C for 1 min (the heating ramp took ~8 sec), (2) cooled the sample to 34°C (the cooling ramp took ~30 sec) and then maintained the sample at 34°C for 2 min. Finally, we quantified bead edges from bright field images by tracing bead edges for every frame using ImageJ and plotting the Feret diameter over time.

Smart’ bead loading
Prior to loading ‘smart beads’, we treated microwell devices with air plasma for 4 min at 150 W plasma (Femto, Diener). Using a syringe pump, we then immediately filled the device with PBST 100 µL/h to expel the air trapped inside each well (for ~10 min) and subsequently flowed PBST at 20 µL/h for 1 h to block the surface. To load beads, we pooled ~10 µL of resuspended solution from each code, washed with PBST one time, and then resuspended in 20 µL PBST. This bead mixture was loaded into the microwell device at 50 µL/h to fill the chamber and then at 10 µL/h for ~2 h in order to fill most of the wells with beads. We then rinsed the device with PBST at 100 µL/h to flush away extra beads before introducing cells.

**T cell staining**

To monitor TCR signaling, Cal-520 AM (AAT Bioquest, Inc.) was used to stain T cells and observe intracellular calcium flux associated with early T cell activation. Briefly, cells were washed, resuspended at 2 million cells/mL in 600 µL of colorless RPMI 1640 (Life technology), 3% (v/v) FBS, 0.02% (w/v) Pluronic F-127 (Sigma-Aldrich, 3% w/v in PBS buffer) and 2 µM Cal-520 (AAT Bioquest, 2 mM in DMSO), and incubated for 40 min at 37°C. During this incubation, cells were resuspended by gently pipetting the solution every 10 min. T cells were then washed with 1 mL colorless RPMI 1640 at RT and resuspended in 60 µL colorless RPMI 1640 with 3% (v/v) FBS at RT prior to loading into the device.

**Cell loading**

During cell staining, the microwell device was washed with colorless RPMI 1640 at 50 µL/h for 40 min at RT. The stained cells were then loaded into the microwell chip at 30 µL/h for 5 min and then incubated for 10 min to allow stained T cells to settle on top of the beads within the microwells. To ensure even cell loading, we then removed the syringe containing the stained cells from the device input, resuspended cells again via repetitive cycles of withdrawing and injection, and then repeated cell loading from the opposite inlet via an identical procedure. Repeating this injection twice allowed most of the beads to be associated with at least one cell. After loading, we washed away unbound cells using colorless RPMI 1640 supplemented with 3% (v/v) FBS at a flow rate of 20 µL/h for 30 min and then incubated at RT for another 30 min. Based on device microwell dimensions, flow rates <50 µL/h only generated drag forces <4 pN, preventing potential force-induced T cell activation during the loading and washing steps; cell loading was also carried out at room temperature to avoid generating any thermo-responsive forces during cell loading.

**Ca²⁺ flux imaging**

Time-lapse imaging experiments were performed on an automated inverted microscope (Nikon Eclipse Ti, Nikon) with a motorized filter turret. For 10-min Ca²⁺ imaging, exposure times were kept 300 ms during all experiments to prevent pixel intensity saturation. Images were acquired at ×4 magnification (S Plan Fluor ELWD 20x Ph1 ADM; Nikon) with 2×2 binning on a sCMOS Andor camera (Zyla 4.2, Andor Technology plc., Belfast, Northern Ireland) using µManager Software. To heat and cool beads, the entire microwell chip assembly was placed on an ITO glass slide mounted on the ASI stage. To exert force on bead-
associated T cells, the chip was heated to and maintained at 37°C for 1 min and then cooled to and kept at 34°C for 2 min. Immediately after cooling, we acquired a total of 150 Ca\(^{2+}\) fluorescence images at 4 s intervals.

‘Smart bead’ imaging

To identify cells within each well, we first imaged the device via bright-field imaging with 2x2 binning using a 4x objective. To identify embedded spectral codes within each bead, we then illuminated the device from above using 292 nm excitation via a Xenon arc lamp (Lambda LS, Sutter Instruments, Novato, CA) equipped with an automated filter wheel (Lambda 10-2, Sutter Instruments, Novato, CA) containing a 292/27 bandpass excitation filter (Semrock, Rochester, NY) paired with UG11 absorptive glass (Newport, Irvine, CA). Emitted light was passed through an additional UV blocking filter mounted within a custom 3D printed holder mounted over the objective and then collected within Ln-channels using nine emission filters (435/40, 474/10, 536/40, 546/6, 572/15, 620/14, 630/92, 650/13, and 780/20 nm). For each image, we then identified all beads and determined the Ln ratios most likely to have produced the observed spectra associated with each pixel via linear unmixing relative to a series of Ln reference spectra as described previously \(^7\). Finally, we created a matrix associating each microwell (indexed by row and column) with the spectral code of the bead within it.

Image analyses

Ca\(^{2+}\) fluxes for individual cells were analyzed in ImageJ. First, we duplicated the full stack of the fluorescence images. For one replicate, we segmented individual cells by finding all local maxima using “Find maxima” in ImageJ with an intensity prominence of 350. For the other replicate, we generated thresholded images using the triangle method and converted to binary images. We then combined the segmented images with the thresholded images using the “AND” operator under image calculator to identify individual cells. For Ca\(^{2+}\) measurements, we: (1) selected a region of interest associated with each cell using the particle analysis tool in ImageJ with a size from 4-pixel units to infinity, and then (2) recorded fluorescence signals and centroids across 150 images. We verified that cells do not move out of ROIs during the course of the experiment for Ca\(^{2+}\) analyses by tracking the centroid of each individual cell using the MultiTracker plugin. We then calculated time-lapse fluorescent intensities for selected ROIs and assigned each to a cell number with unique \(x\) and \(y\) coordinates. Signals were analyzed in Matlab (MathWorks) by custom written scripts and can be provided upon request. Briefly, we: (1) extracted the spatial coordinates of centers of individual microwells from the bright-field image, (2) converted spatial coordinates to microwell row and column numbers, (3) assigned each cell to a specific microwell by comparing cell and microwell centroids and assuming a 7.5 \(\mu\)m well diameter, and then (4) associated traces for each cell with the embedded code (and thus the peptide sequence) of the bead present within that microwell.

Ca\(^{2+}\) traces were normalized for each cell by plotting the fluorescence ratio at each time point divided by the measured intensity at time zero. Integrated Ca\(^{2+}\) signals were calculated by subtracting 1 from each
timepoint and integrating Ca\(^{2+}\) traces over 10 min; all cells with an integrated Ca\(^{2+}\) signal > 0 were considered ‘positive’ cells.

**Bootstrap Hypothesis Testing**

To calculate bootstrapped p-values for each peptide tested using BATTLES, we iteratively: (1) pooled integrated Ca\(^{2+}\) signals of all peptides, (2) calculated the number of measurements associated with the peptide of interest (n) and for this entire pool (m), (3) sampled n and m observations with replacement from the merged pool, and (4) calculated the difference (\(t^*\)) between the mean of the first n observations and mean of the second m observations. In each case, each observation was the fold-change of a particular integrated Ca\(^{2+}\) signal relative to the mean Ca\(^{2+}\) signal across all measurements. We repeated this procedure 100,000 times and then estimated the probability that an observed distribution was statistically significantly different from the pooled distribution as follows:

\[
p-value = \frac{\text{number of times}\{t^* > t_{\text{obs}}\}}{100,000}
\]

For TCR589 and TCR55 cells interacting with 21 peptides, we applied a Bonferroni correction for multiple hypothesis testing at a significance of 0.05 (0.05/21 = 0.0024). For concentration multiplex experiments, we pooled specific peptides at a given concentration with the same peptide at other concentrations for bootstrap hypothesis testing and therefore determined a Bonferroni-corrected significance threshold of p = 0.013 (0.05/4). For DMF5 T cell interacting with two different peptide classes, we pooled all 5 peptides within each class plus the Tax peptide and therefore determined a Bonferroni-corrected significance threshold of p = 0.008 (0.05/6).

**Declarations**

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**Author contributions**
Y.F. conceptualized the platform and validation experiments; X.Z. made all the T cell lines and UV-labile pMHC folding. Y.F., X.Z., and A.K.W. analyzed data; K.C.G and P.M.F. provided funding, resources, mentorship, and project supervision. Y.F., X.Z., A.K.W., K.C.G. and P.M.F. wrote the paper.

DECLARATION OF INTERESTS

Stanford University and Chan Zuckerberg Biohub have filed a provisional patent application (U.S. Provisional Patent application No. 63/108,162) on the BATTLES technology described here, and Y.F., X.Z., A.K.W., K.C.G. and P.M.F. are named inventors.

Data and code availability

AutoCAD designs (parallel flow focuser and microwell device) and T cell Ca^{2+} flux data has been deposited to OSF repository ([https://osf.io/xs7zf/](https://osf.io/xs7zf/)).

References


Figures
Figure 1

Overview of BATTLES assay and ‘smart beads’. (a) BATTLES applies shear forces to pMHC/TCR interactions at cell surfaces via thermally responsive ‘smart beads’ that are spectrally encoded (with a 1:1 linkage between embedded spectral code and presented pMHC sequence, presented pMHC density, or the magnitude of the applied load). (b) By loading T cells onto the surfaces of ‘smart beads’ loaded into microwell arrays, BATTLES allows for high-throughput single-cell measurement of T cell responses (cellular Ca2+ flux) via microscopy after the application of force. (c) Schematic and formula detailing how thermal bead expansion applies force to pMHC/TCR interactions at cell surface; \( R_{\text{cell}} \) is the T cell radius (~4 \( \mu \)m), \( \kappa \) is the modulus of rigidity of the ‘smart bead’, \( R_{\text{bead}} \) is the radius of the bead prior to heating, and \( \Delta R_{\text{bead}} \) is the change in bead radius upon cooling from 37°C to 34°C. Force ramps are calculated assuming beads swell over 60 s after cooling. (d) Cartoon schematic of polymeric ‘smart bead’ matrix. (e) Representative bright field images showing the change in radius for a ‘smart bead’ upon heating (25 to 37 °C) and cooling (37 to 34 °C). Scale bar: 25 \( \mu \)m. (f,g) Measured bead radii (f) and changes in radii (g) over time for 12 beads upon heating and cooling. (h) Calculated expansion forces for 12 beads.
‘Smart beads’ can display 21 different peptide sequences at physiological densities. (a) Image of 3 different Lns (Eu, Dy, and Sm) upon excitation with a UV LED (285 nm). (b) Cartoon schematic showing 2 example ratiometric codes. (c) Measured median Ln ratios (colored markers) for 872 beads containing equal amounts of Eu and 21 different combinations of Sm and Dy along with the expected target ratios. (d) Structural schematic and amino acid frequency per position after selection with TCR55 in yeast display experiments; blue and orange residues denote TCR and MHC anchor contacts, respectively. (e) Sequences of HIVpol-derived peptides coupled to ‘smart beads’ containing particular spectral codes. (f) Schematics detailing UV-exchange method to generate biotinylated pMHCs bearing desired peptides (left) and coupling of biotinylated pMHCs to ‘smart beads’ bearing streptavidin crosslinked to carboxyl groups via EDC chemistry (right). (g) Schematics detailing the quantification of the interfacial pMHCs via smTIRF microscopy using fluorescently labeled antibodies to detect peptide-loaded MHCs. (h) smTIRF images for an example pMHC-coated bead (code 8, +pMHC) and an example negative control bead (-pMHC). White dashed circles denote the bead circumference settled on the slide (diameter ~ 50 µm); yellow circles denote the size of an average T cell; numbers indicate identified pMHCs within yellow circles. (i) Estimated surface density (left) and number of pMHCs/cell (right) from surface density
measurements across all ‘smart bead’ codes; markers denote means and error bars denote standard deviation. The dashed line represents the physiological number of pMHCs per cell54.

**Figure 3**

Activation responses for TCR589-transduced T cells interacting with 21-peptide library. (a) Cartoon schematic of BATTLES workflow and expected results. (b) Representative bright field images of the 1440 well microwell device containing ‘smart beads’ and loaded T cells. (c) Representative merged lanthanide and fluorescence images of ‘smart beads’ and associated T cells in the presence of a Ca2+-sensitive dye. (d) Representative images (left) and processed intensity traces (right) quantifying Ca2+ flux within single TCR589 T cells interacting with pMHC-coated ‘smart beads’ bearing the stimulatory HIVpol peptide (IPLTEEAEL) (top) and a nonstimulatory peptide (VPLTEDAEL) (bottom). (e) Ca2+ signal intensity as a function of time for all positive single cells (\( \int (I/I_0-1) > 0 \)) (light grey); mean signal intensity over time is shown in black. (f) Integrated Ca2+ signals for all positive single cells as a function of peptide sequence. Individual cell signals are shown as black markers; box lower and upper limits indicate 25th and 75th percentiles, respectively. Grey squares represent the mean values. (g) Estimated p-value (calculated via bootstrapping, see Methods) vs. the log2-transformed mean fold change for integrated Ca2+ signals for each peptide sequence. Grey box indicates Bonferroni-corrected p-value at a significance of 0.05 (p = 0.0024). (h) Integrated Ca2+ signals for each peptide across 2 replicates. Markers indicate mean; error bars indicate SEM; dashed black line indicates the 1:1 line; red dashed line indicates a linear regression.
Figure 4

Activation responses for TCR55-transduced T cells interacting with 21 peptide library. (a) Schematic illustrating previously observed divergent cellular reactivity responses for TCR55 vs. TCR589 interacting with the HIVpol-derived peptide. (b) Integrated Ca2+ signals for positive cells across all peptide sequences tested. Integrated signals for individual cells are shown as black markers; box lower and upper limits represent 25th and 75th percentiles, respectively. Grey squares represent the mean values. (c) Estimated p-value (calculated via bootstrapping, see Methods) vs. the log2-transformed mean fold change for integrated Ca2+ signals for each peptide sequence; Grey box indicates Bonferroni-corrected p-value at a significance of 0.05 (p = 0.0024). (d) Integrated Ca2+signals for each peptide across 2 replicates. Markers indicate mean; error bars indicate SEM; dashed black line indicates the 1:1 line; red dashed line indicates a linear regression.
Figure 5

Multiplexing sequence, applied force, and displayed pMHC densities using BATTLES. (a) Typical catch/slip bond lifetime profiles. (b) Schematic and images indicating that change in bead radius depends on the amount of sodium acrylate (SAc) within the ‘smart bead’ matrix. (c) Measured changes in bead radius upon cooling from 37°C to 34°C for 3 codes (9, 6, and 6 beads/code) containing different SAc concentrations. (d) Calculated expansion forces for ‘smart beads’ in c based on measurements of bead radii, changes in bead radii, and the rigidity of the NIPAM slab. (e) Mean integrated Ca2+ signals for positive cells interacting with VPLTEDALL, VPITEDSQL, VPLTEDAEL and IPLTEEAEL peptides at low (~15 pN/s), moderate (~25 pN/s) and high (~55 pN/s) force ramps. Zero force data is from a TCR55 control experiment in which temperature was maintained at 34°C. (f) Schematic indicating loss of TCR specificity at higher pMHC concentrations. (g) Mean integrated Ca2+ signals for positive cells interacting with VPLTEDALL, VPITEDSQL, VPLTEDAEL and IPLTEEAEL peptides at 1X, 7X and 27X pMHC concentrations. (h) Summarized integrated Ca2+ signals for four selected peptides under three pMHC concentrations. (i) Estimated p-value (calculated via bootstrapping, see Methods) vs. log2-transformed mean fold-change of integrated Ca2+ signals for 4 peptides at 3 different pMHC concentrations; grey area represents p-value>0.013 (Bonferroni-corrected p-value at a significance of 0.05). Error bars in e, g and h indicate SEM.
Figure 6

Activation responses for the therapeutically-relevant DMF5 TCR system interacting with 11 peptide sequences from 2 classes. (a) Schematic illustrating that DMF5 TCR shows comparable cellular reactivity to two different classes of peptide sequences (GIG and DRG, respectively) in prior co-culture assays. (b) Integrated Ca2+ signals for positive DMF5-transduced T cells interacting with Tax (LLFGYPVV, a ‘negative’ control peptide identified from coculture experiments) and 10 additional peptides from the GIG (grey) and DRG (red) classes. Integrated signals for individual cells are shown as black markers; box lower and upper limits represent 25th and 75th percentiles, respectively; grey squares represent mean values. (c) Estimated p-value (calculated via bootstrapping, see Methods) vs. log2-transformed mean fold change of integrated Ca2+ signals; grey area represents p-value > 0.05. (d) Mean integrated Ca2+ signals for positive DMF5 cells for each peptide across 2 technical replicates. Error bars indicate SEM; dashed black line indicates the 1:1 line; red dashed line indicates linear regression.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MovieS1.mp4
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- MovieS5.mp4
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