Mechanosensing in T Lymphocyte Activation

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Supporting Material

Materials and Methods

Cell isolation

T cells were isolated from C57BL/6 mouse lymph nodes using bead-based negative selection (Dynal beads, Invitrogen), yielding populations of > 85% naïve CD4+ cells. Purified cells were resuspended in RPMI 1640 media supplemented with 10% fetal bovine serum, 10mM HEPES buffer, 2mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomyocin, and 50 μM 2-Mercaptoethanol (Sigma Aldrich) and used immediately in experiments as described below. Unless otherwise specified, all reagents were purchased from Invitrogen.

Substrates and protein preparation

Polyacrylamide gels were prepared following established methods (1). Gels rigidity was controlled by varying the amount of bisacrylamide crosslinker while holding the total acrylamide concentration constant at 10%. Bisacrylmaide concentrations of 0.8, 0.4, 0.1, 0.05, 0.02, and 0.01 % (w/v) produced substrates with bulk Young's modulus of 200, 100, 25, 10, 5, and 2 kPa, respectively, which was verified using established AFM-indentation methods (2). Acrylamide-modified streptavidin (Invitrogen Life Sciences) was polymerized into the gel to allow subsequent tethering of biotinylated antibodies. Acrylamide-streptavidin was incorporated at 667 μg/ml for the 200, 100, and 25 kPa gels, 267 μg/ml for the 10 kPa gels, and 133 μg/ml for the 5 and 2 kPa gels. These different concentrations were chosen to normalize the surface concentration of immobilized antibodies. To compare these surface concentrations of captured protein, biotinylated antibodies were co-labeled using an amine-reactive Alexa 568 dye (Invitrogen) and then imaged by fluorescence microscopy. Epifluorescence microscopy revealed a lateral distribution of antibodies (red, Fig. S1A) that was highly uniform. Typical CD4+ T cells (green) are included in this image for comparison of scale. Confocal microscopy (Fig. S1B) showed that these antibodies were confined to a thin (3-4 μm) upper layer of the gel, characterized by a sharp increase in fluorescence within the first micrometers followed by a more gradual decrease with depth into the gel. These overall profiles were similar across compositions, and the peak fluorescence intensity observed across the profile was similar for each of the different preparations. The surface concentration of antibodies was estimated by fluorescence microscopy using a 20x objective with 1.6 μm depth of field. For a given concentration of acrylamide-streptavidin, the measured fluorescence was significantly brighter on gels of reduced crosslinking, potentially a result of higher porosity and surface accessibility associated with these substrates. Consequently, the concentration of acrylamide-streptavidin was adjusted for each gel composition to normalize the surface density of tethered antibodies (Fig. S1C). Activating antibodies against CD3 (epsilon subunit, clone 145-2C11, eBioscience) and CD28 (clone 37.51, eBioscience) were biotinylated using a hydrazide-based chemistry (EZ-Link* Hydrazide-LC-Biotin, Thermo Scientific) that targets carbohydrate moieties. For co-presentation on the same surface, anti-CD3 and anti-CD28 antibodies were mixed at a 1:1 ratio for a total concentration of 30 μg/ml then incubated with prepared substrates for 45 minutes at 37^0C and 5% CO2. For experiments in which anti-CD3 and anti-CD28 are presented on different surfaces, gels were incubated with one of the antibodies at a concentration of 30 μg/ml, while the other was immobilized onto polystyrene beads of 4-

μm diameter (sulfate surface chemistry, Invitrogen) following the manufacturer's instructions. Briefly, beads were washed in PBS to remove azide and tween solution then incubated with anti-CD3/CD28 at a concentration of 20 μg/ml overnight. Beads were then washed with PBS and incubated for 1 hour with 4% bovine serum albumin (BSA). In these experiments, cells were allowed to adhere onto the gels for 30 minutes and then exposed to an excess of beads (3:1 ratio of beads:cells) carrying the other protein.

Assays of cell function

Unless otherwise stated, naïve T cells were seeded onto prepared surfaces at a concentration of 3,400 cells/mm^2 in fully supplemented RPMI media and maintained under standard cell culture conditions. IL-2 secretion was assayed using a commercially available kit (Miltenyi Biotec) as previously described (3, 4). Briefly, cells were incubated with an IL-2 capture reagent (part of the assay kit) immediately prior to seeding. After 1 hour, the media was exchanged with pre-warmed, fully supplemented RPMI media. After a total of 6 hours (or 16 hours for select experiments indicated in the main text) cells were incubated with an APC-conjugated, detection antibody against IL-2 (a second component of the detection kit). Cells were then fixed and imaged by fluorescence microscopy. IL-2 intensity was quantified from low magnification (20x objective, 1.6 μm depth of field, 400 μm field of view) images. Total fluorescence was measured on a cell-by-cell basis. All images from a single experiment were collected at the same microscopy session. Cell attachment density was measured by phase contrast microscopy six hours after seeding, unless otherwise stated. For experiments examining the effect of myosin contractility on IL-2, blebbistatin was added to the culture media at the 1 hour timepoint to a final concentration of 100 μM. For characterization of Zap70 and SFK phosphorylation 30 minutes after initiation of cell-substrate contact, blebbistatin was included in the culture media from the beginning of the experiment.

T cell activation, like other cellular functions, is cooperative and a function of the local cell density. This raises the possibility that IL-2 secretion is directly related to the per-area concentration of cells and not substrate rigidity, particularly on the 10 kPa gels which exhibited reduced cell attachment relative to the other surfaces. To address this possibility, assays of cell function were repeated at increasing seeding densities on the 10 kPa gels. Cell attachment density was not linearly related to seeding density, reflecting cooperativity between these cells. Increasing cell seeding density by a factor of 50% raised cell attachment density by 2.5 fold, slightly higher than the attachment observed on the 25 kPa gels. IL-2 secretion, however, increased a modest and not statistically significant amount, 22±39% ($P < 0.51$, mean±s.d., n=7), much less than the 2 fold difference in response observed as gel rigidity changed. A further increase in cell seeding density to 10,200 cell/mm^2 (3-fold higher) induced a 1.9 fold increase in IL-2 secretion but was associated with a 5-fold increase in cell attachment, higher than that observed on the stiffest surfaces. We thus conclude that while cell-cell cooperativity may contribute to increased activation, it is not the major contributor to the effects seen here as a function of substrate properties.

Similarly, it is recognized that T cell activation can be sensitive to surface ligand concentration. Reducing the concentration of acrylamide-streptavidin used to prepare the 10 kPa gels, which was done to normalize the resultant per-area concentration of captured

antibody, may have thus influenced cell response. To test this, 10 kPa surfaces were prepared with 667 μg/ml acrylamide-streptavidin (the concentration used on the stiffer gels) which accordingly raised the surface concentration of anti-CD3 and anti-CD28. Compared to surfaces prepared at the adjusted concentration of 267 μg/ml, 6-hour IL-2 secretion exhibited a slight but not statistically significant increase, 13±26% ($P < .26$, mean±s.d., n=3). Cell attachment density also exhibited a small but not statistically significant increase, 8±17% ($P < .88$, mean±s.d., n=3). We conclude that the loss of cell response observed on the 10 kPa gels was not a result of the changing the acrylamide-streptavidin concentration used to prepare that gel.

Statistics

For assays of IL-2 secretion and Lck/Zap70/Pyk2 phosphorylation, fluorescence intensity was measured from at least 500 cells for each condition. The average fluorescence intensity from these cells was used to represent each condition, and experiments were carried out at least three independent times. All data were analyzed using standard one- and two-way ANOVA techniques in conjunction with Tukey HSD multiple comparison approaches.

Immunostaining

Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton-X solution, washed in PBS buffer, and labeled with the following phospho-specific antibodies: Lck (phospho-Tyr 393, Novus Biologicals), Zap70 (phospho-Tyr 493, Cell Signaling), and Pyk2 (phospho-Tyr 580, Life Technologies). Samples were then stained with secondary antibodies and phalloidin as specified in the text, using standard techniques. Cellular levels of phosphorylated proteins were measured and analyzed for statistical significance using methods identical to the IL-2 secretion assay described above.

Supporting References

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2. Costa, K. D., and F. C. Yin. 1999. Analysis of indentation: implications for measuring mechanical properties with atomic force microscopy. J Biomech Eng 121:462-471.
3. Shen, K., V. K. Thomas, M. L. Dustin, and L. C. Kam. 2008. Micropatterning of costimulatory ligands enhances CD4+ T cell function. PNAS 105:7791-7796.
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Supporting Figures.

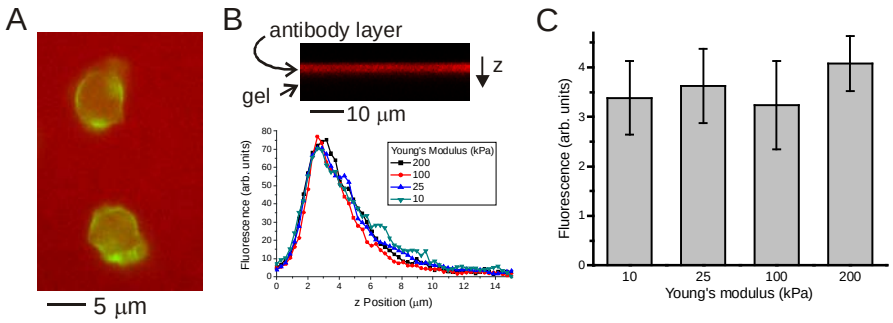

Figure S1. Distribution of surface-tethered antibodies. Anti-CD3 and anti-CD28 antibodies were labeled with both biotin (carbohydrate-targeting) and Alexa 568 (red, amine-targeting) before tethering to prepared gels. (*A*) These antibodies formed a uniform layer across the gel surface. Adherent T Cells (labeled with phalloidin) are shown in green and included as a size reference. This image illustrates cells on a 200 kPa gel. (*B*) *top*, Antibodies were confined to a thin layer of the gel surface, as illustrated in this side projection of a 200 kPa gel collected by confocal microscopy. *bottom*, Depth profiles of antibodies were similar across gel compositions. (*C*) No difference in fluorescence intensity was observed across gels of different rigidity ($\alpha = 0.05$, ANOVA, n=6. Data are mean ± s.d.).

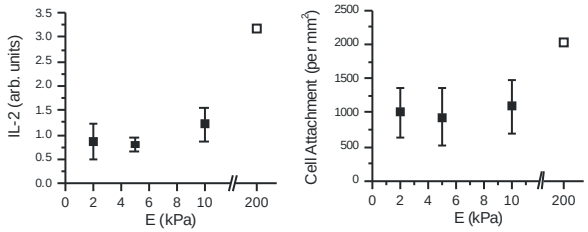

Figure S2. T cell activation on substrates of lower Young's modulus. Data of IL-2 secretion and cell attachment density on surfaces of 10, 5, and 2 kPa are denoted by closed squares, indicating mean ± s.d., n=3. No significant difference in either measure was observed across these three preparations. The open squares indicate mean IL-2 secretion and cell attachment observed on 200 kPa reference surfaces carried out in parallel with the softer surfaces.

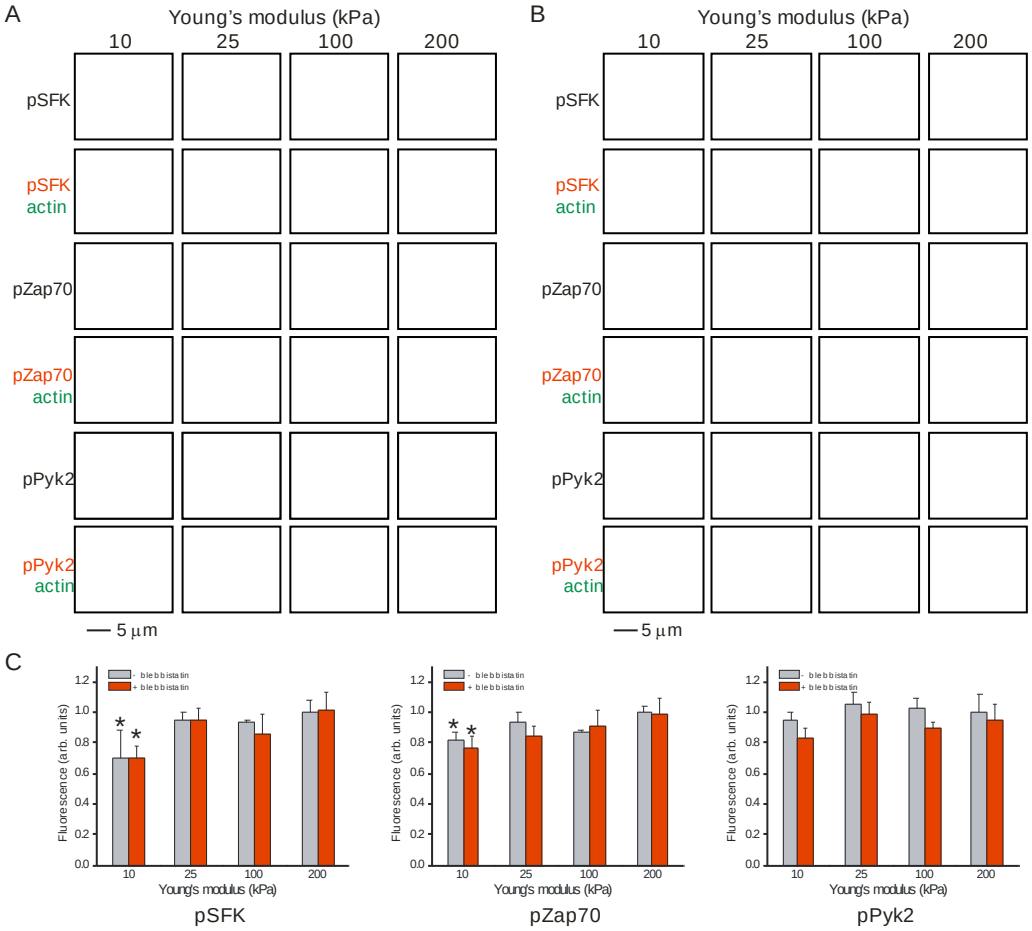

Figure S3. Sustained Lck, Zap-70, and Pyk2 phosphorylation as a function of substrate rigidity. (*A*,*B*) Distribution of phosphorylated proteins in cells 30 minutes after initiation of contact with the substrates in the (A) absence and (B) presence of 100 μM blebbistatin. (*C*) Quantitative comparison of whole-cell fluorescence associated with each phosphorylated protein. Data are mean ± s.d., n=3. * $P < 0.05$ compared to 200 kPa, non-blebbistatin surface.