Supplemental Data

THE αβ T CELL RECEPTOR IS AN ANISOTROPIC MECHANOSENSOR

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Running Title: TCR Quaternary change and signaling

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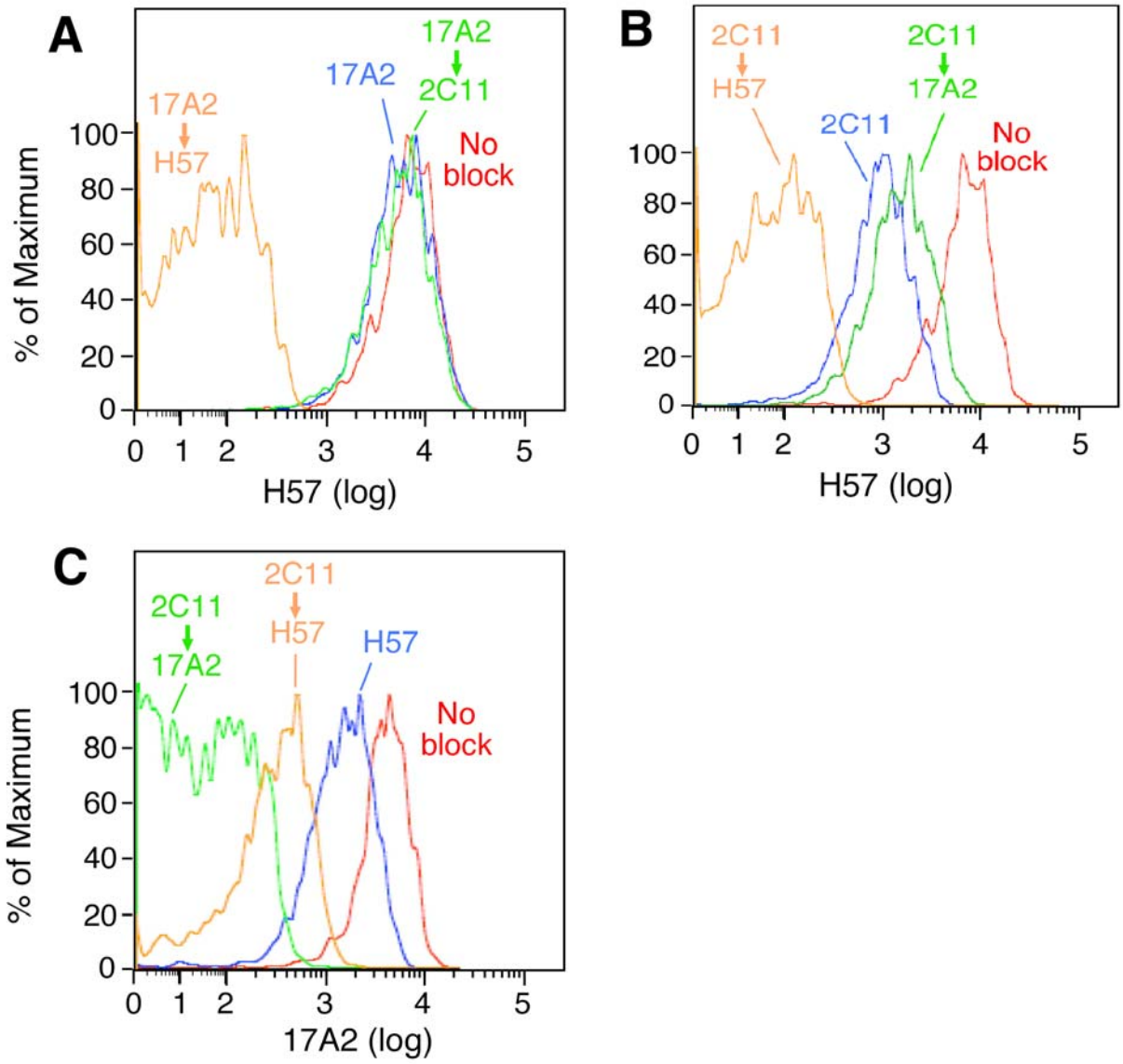

Figure S1. Antibody Crossblocking on the TCR Complex Expressed on the Surface of T-lineage Cells

Thymocytes were isolated from C57BL/6 mice and pre-incubated with the indicated, unlabeled first Fab fragment [17A2 (A), 2C11 (B), and H57 (C)] for 20 min before addition of the second Fab fragment (shown beneath the arrows). Following sequential blocking, fluorochrome-labeled H57 (A and B), or 17A2 (C) staining levels were monitored using flow cytometry. The CD4 single-positive population was gated for analysis. Data are representative of three experiments.

	Fluorescence Intensity (FI) at 10 ug/ml (excitation at 485 nm, emission at 535 nm)
17A2-FITC	12301
2C11-FITC	12208

FI (17A2-FITC) ≈ FI (2C11-FITC)

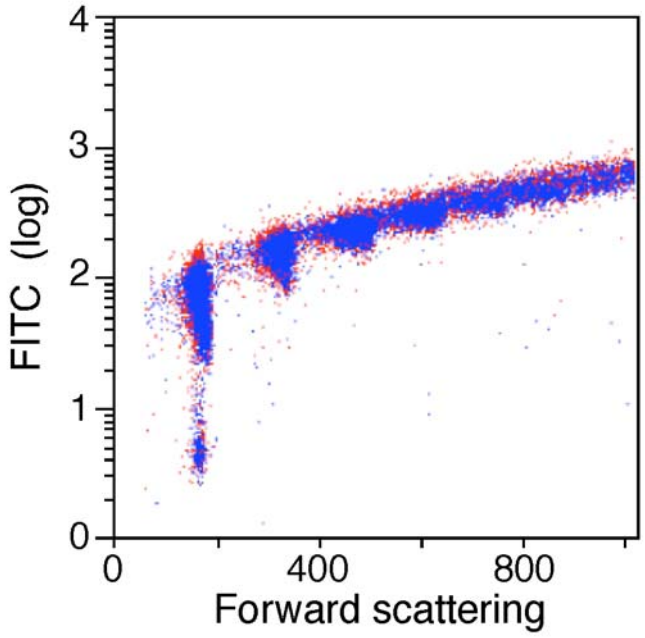

Figure S2. Antibody Immobilization on Protein G Coupled Magnetic Beads

We measured FITC labeling ratios in 17A2 and 2C11 using a VICTOR3™ multi-label reader (PerkinElmer). Each magnetic bead preparation with immobilized 2C11-FITC or 17A2-FITC has the same set of MFI values (Red: 2C11, Blue: 17A2).

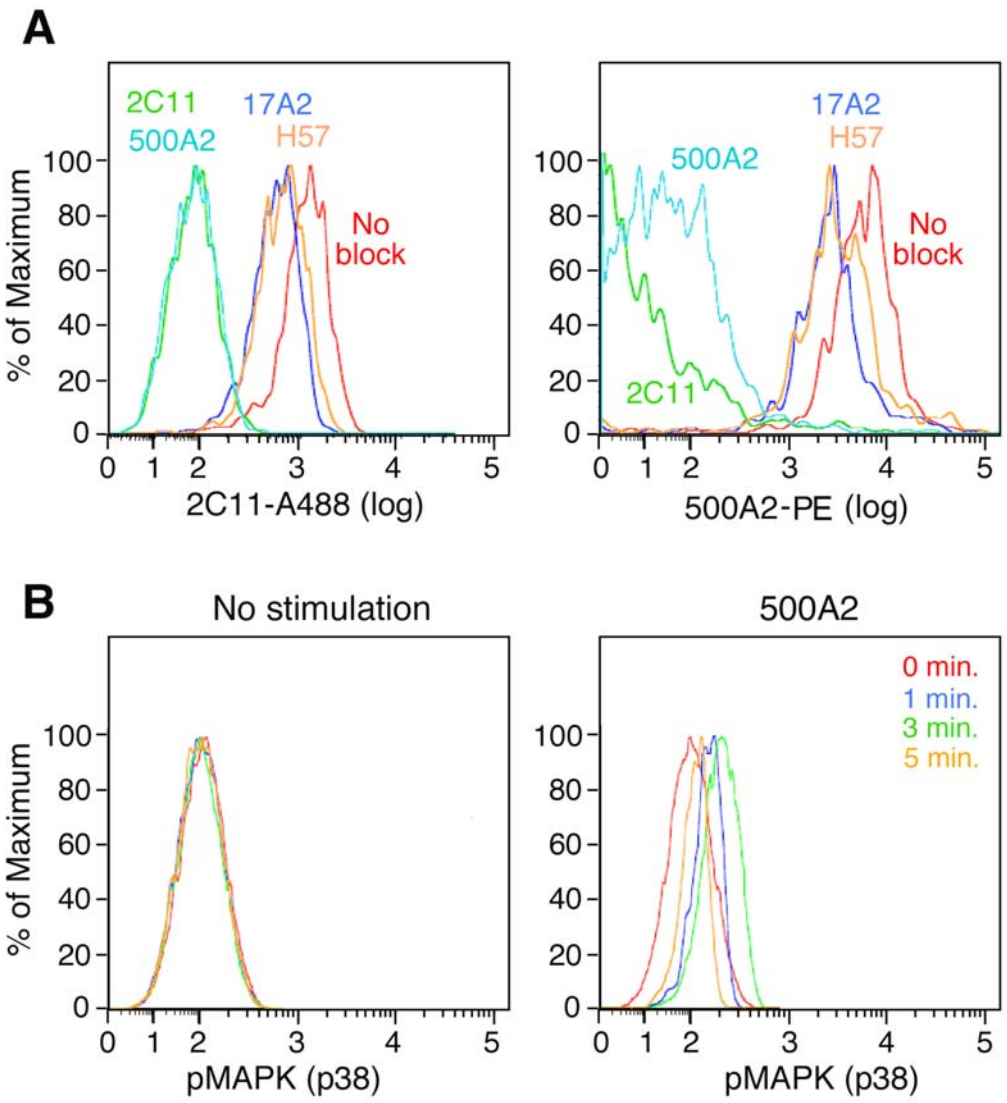

Figure S3. 500A2 Binding and Activation

(A) Binding competition pattern of 500A2 appears the same as that of 2C11. LNs were isolated from C57BL/6 mice. mAbs against T cell surface markers, including CD3, were stained for flow cytometric analysis. Each histogram represents 2C11 (left panel) and 500A2 (right panel) binding after various unlabeled antibody blocking. CD4 single-positive T cells were gated for analysis. (B) Phosphorylation state of MAPK was determined by intracellular staining using BD Phosflow Lyse/Fix buffer BD and Phosflow Perm Buffer III after incubation with immobilized 500A2 (500A2) or left unstimulated (No stimulation). Data are representative of three experiments.

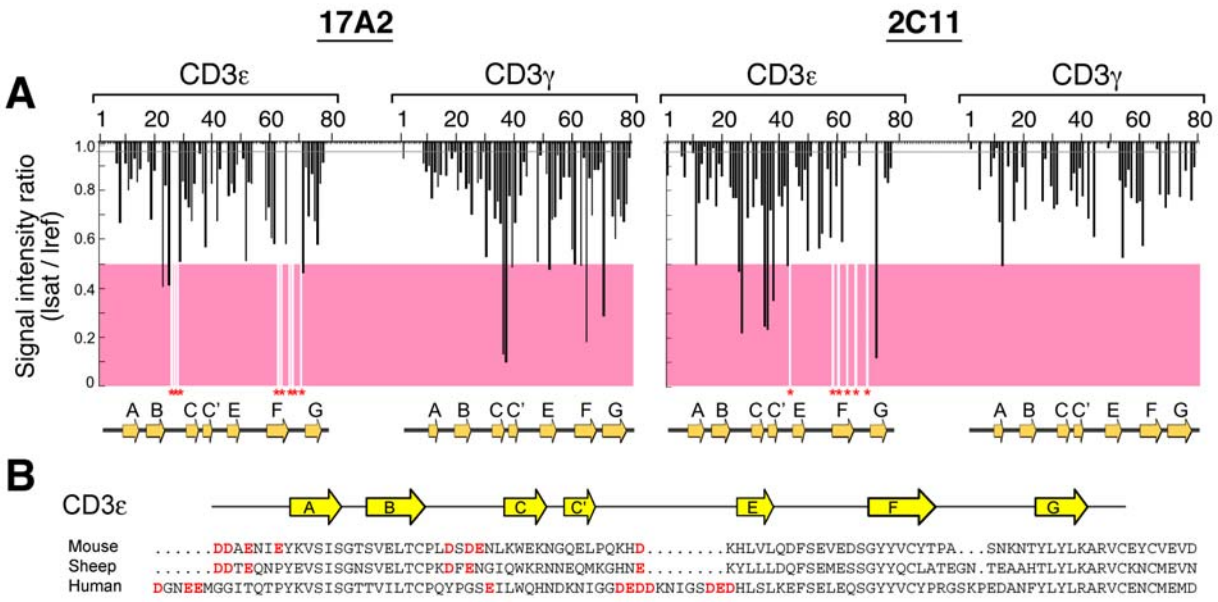

Figure S4. Summary of Signal Reduction in Cross-saturation Experiments Induced by 17A2 and 2C11 Binding

(A) Intensity reduction ratio for each resonance in the cross-saturation experiment. Irradiation was applied to the methyl proton resonances of the Fabs for 0.5 sec. The pink shading indicates the residues that are mapped in Figure 3A. The residues which disappear from their original positions upon Fab binding are indicated by white bars with red dots. (B) Multiple sequence alignment of CD3ε from different species. Typical distributions of negatively charged amino acids in CD3ε are highlighted in red.

A Heavy Chain

	FW1	**CDR1**	FW2	**CDR2**
2C11	EVQLVESGGGLVQPGKSLKLSCEASGFTFS	**GYGMH**	WVRQAPGRGLESVA	**YITSSSINIKYADAVKG**
17A2	EVKLVESGGGLVQPGGSLRLSCAASGFTFS	**NFPMA**	WVRQAPKKGLEWVA	**SISSGGGGTFYPDSVKD**

	FW3	**CDR3**	FW4
2C11	RFTVSRDNAKNLLFLQMNILKSEDTAMYYCAR	**FDWDKNY**	WGQGTMVTVSS
17A2	RFTISRNNAKNTLYLQIDSLRSEDTASYYCVR	**PQGGFAS**	WGQGTLVTVSS

B Light Chain

	FW1	**CDR1**	FW2	**CDR2**
2C11	QMTQSPSSLPASLGDRVTINC	**QASQDISNYLN**	WYQQKPGKAPKLLIY	**YTNKLAD**
17A2	VMTQSPKSMSISIGDRVTMNC	**KASQNVINYIA**	WYQQKPGQSPKLLIY	**YASNRYT**

	FW3	**CDR3**	FW4
2C11	GVPSRFSGSGSGRDSSFTISSLESEDIGSYYC	**QQYYNYPWT**	FGPGTKLEIKR
17A2	GVPDRFTGGGSGTDFTLTINSVQAEDAAFYYC	**QRIYNSPWT**	FGGGTKLELKR

Figure S5. Amino Acid Sequence Comparison of 17A2 and 2C11 Variable Regions

Framework regions (FW) and complementary determining regions (CDR) are denoted by Chothia's methods.

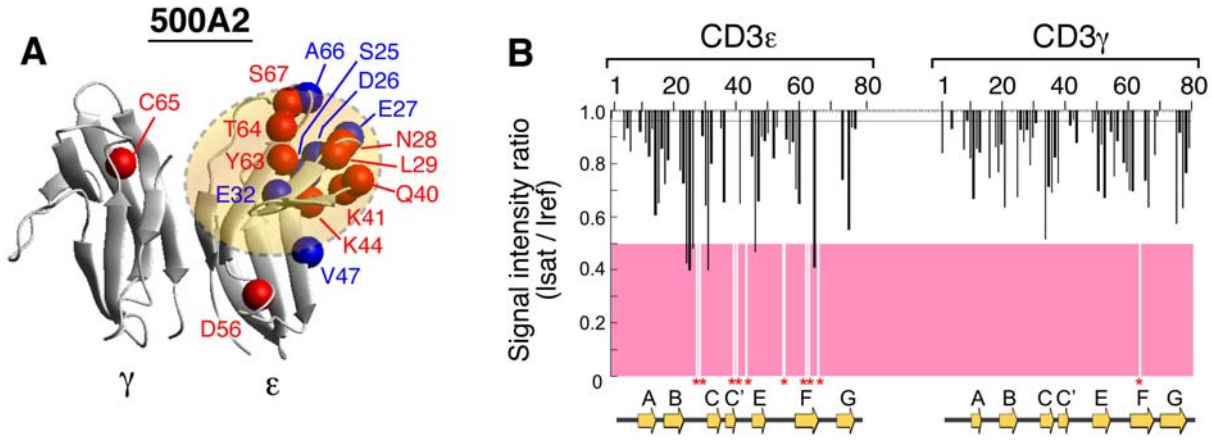

Figure S6. Mapping of 500A2 Binding Surface Determined by Cross-saturation and Chemical Shift Perturbation Experiments

(A) Mapping of the residues affected by cross-saturation experiments. Blue spheres indicate residues that experience significant cross-saturation (signal reduction <0.5) indicating direct contact with the Fabs. Red spheres indicate the residues that disappear upon Fab binding. The major binding surface area is shaded by the brown ellipsoid. (B) Intensity reduction ratio for each resonance in the cross-saturation experiment. Irradiation was applied to the methyl proton resonances of the Fabs for 0.5 sec. The pink shading indicates the residues that are mapped in the panel A. Residues that disappear from their original position upon Fab binding are indicated by white bars with red dots.

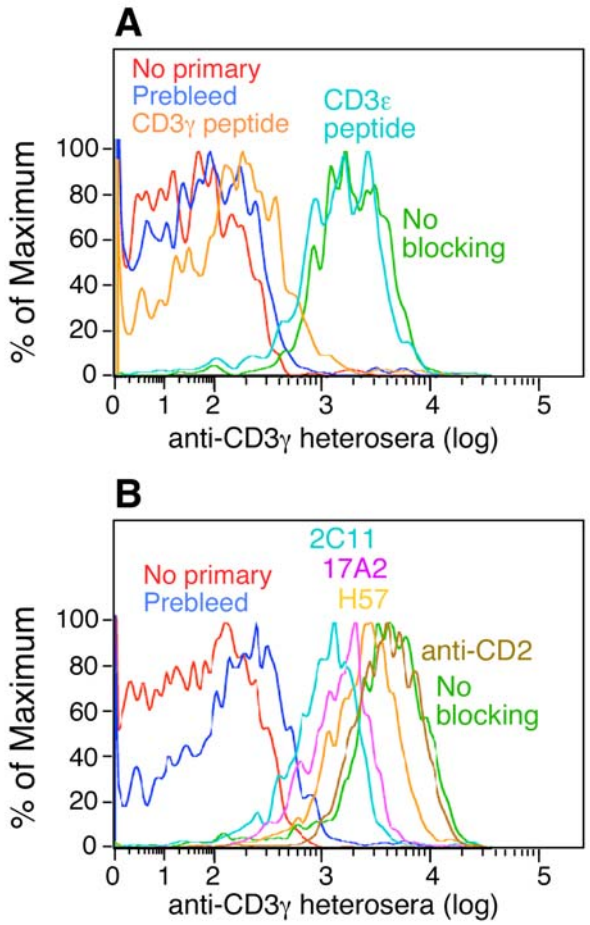

Figure S7. Anti-CD3γ N-terminus-specific Heterosera Binding on T Cells

(A) Anti-CD3γ heterosera specific for the N-terminus of murine CD3γ was added after preincubation with an excess amount (10 μg/ml) of N-terminal CD3γ peptide (QTNKAKNLVC) or N-terminal CD3ε peptide (DDAENIEYC). After 20 min incubation with 1/200 diluted sera, we stained with 10 μg/ml of anti-rabbit IgG-FITC for detection. Reagents and conditions used are given. (B) After preincubation with H57, 2C11, 17A2, or anti-CD2 antibody (50 μg/ml), the staining level of a heterosera recognizing the N-terminus of CD3γ was measured by flow cytometry. The histogram suggests that the N-terminus of CD3γ resides close to the H57, 2C11, 17A2 binding region involving the TCRCβ FG loop and CD3εγ heterodimer. A CD4 single-positive thymocyte population isolated from C57BL/6 mice was gated for the analysis. Data are representative of three experiments.

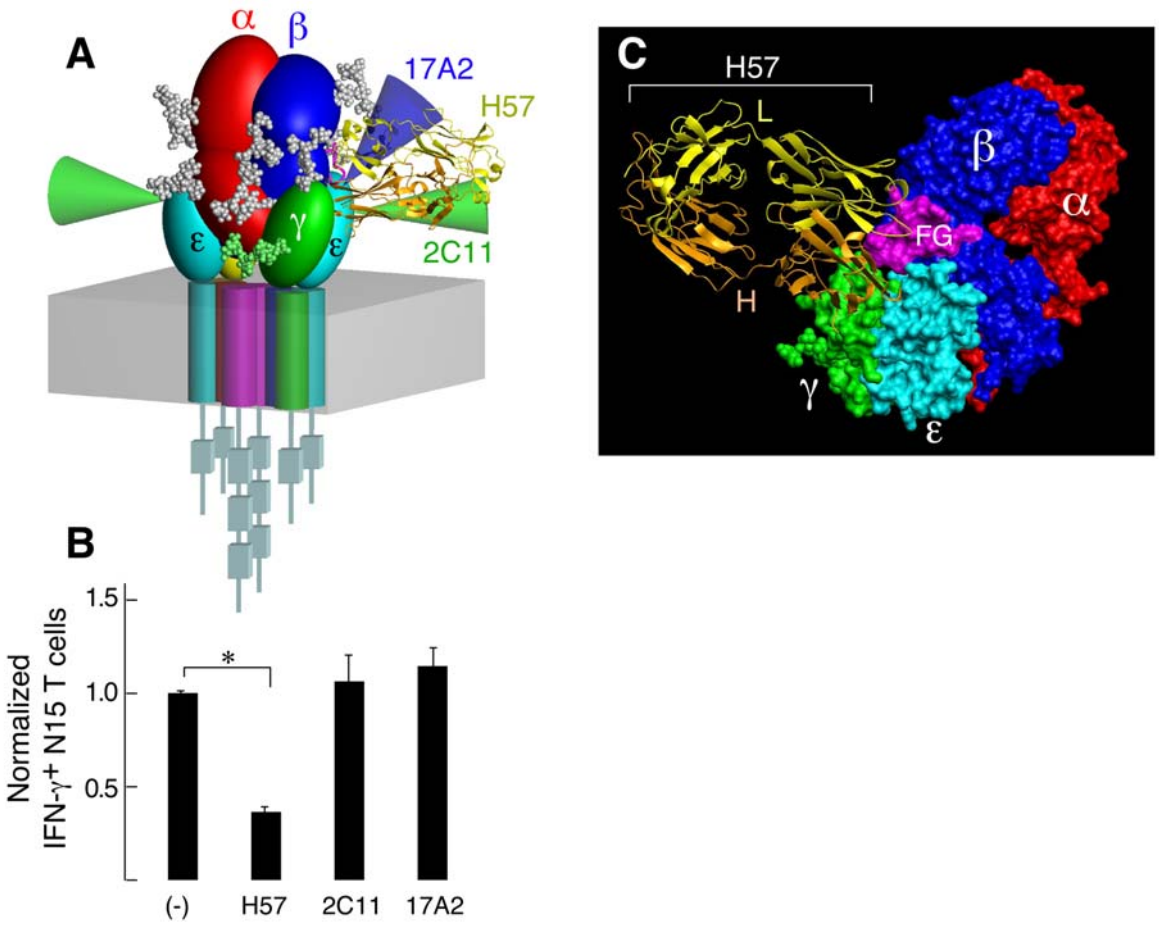

Figure S8. pMHC-triggered αβTCR-mediated Signaling via the TCRβ-CD3εγ Module. (A) Schematic drawing of the binding of Fabs to the TCR complex. 17A2 and 2C11 positions are given by blue and green cones, respectively. The Cβ FG loop-specific H57 Fab binding is represented as a ribbon diagram. CD3γ and its glycan are in green; CD3ε is in light blue, and α and β glycans are shown in gray. CD3δ is in yellow and CD3ζ is in pink. The cell membrane is schematically represented in gray with TM segments (cylinders) and ITAMs (rectangles) in the cytoplasmic tail. (B) N15 T cells from N15TCRtgRAG2$^{-/-}$ mice were stimulated with VSV8 (0.1 nM) peptides after first blocking with saturating amounts of Fab fragments (20 μg/ml). Following VSV8 peptide stimulation, IFNγ production was monitored by intracellular staining and the relative number of cytokine-producing cells normalized to the Fab-untreated control (-). Two-sided exact Wilcoxon rank-sum test was used to compare. Values are

average ± SD (n=4). *p≤0.05 (C) A docking model depicts interactions between the H57 Fab (ribbon) and CD3εγ in the TCR complex (space filling representations). H57 binding to the N15αβ heterodimer was taken from the structure by Wang et al. (42). The overall topology is shown from the TCRβ side using a quaternary structural model rotated ~90° about the Y-axis relative to panel A. The H57 Fab is predicted to interact with the CD3εγ heterodimer plus the TCRαβ heterodimer, unlike CD3εγ–only binding 17A2 or 2C11 Fabs.

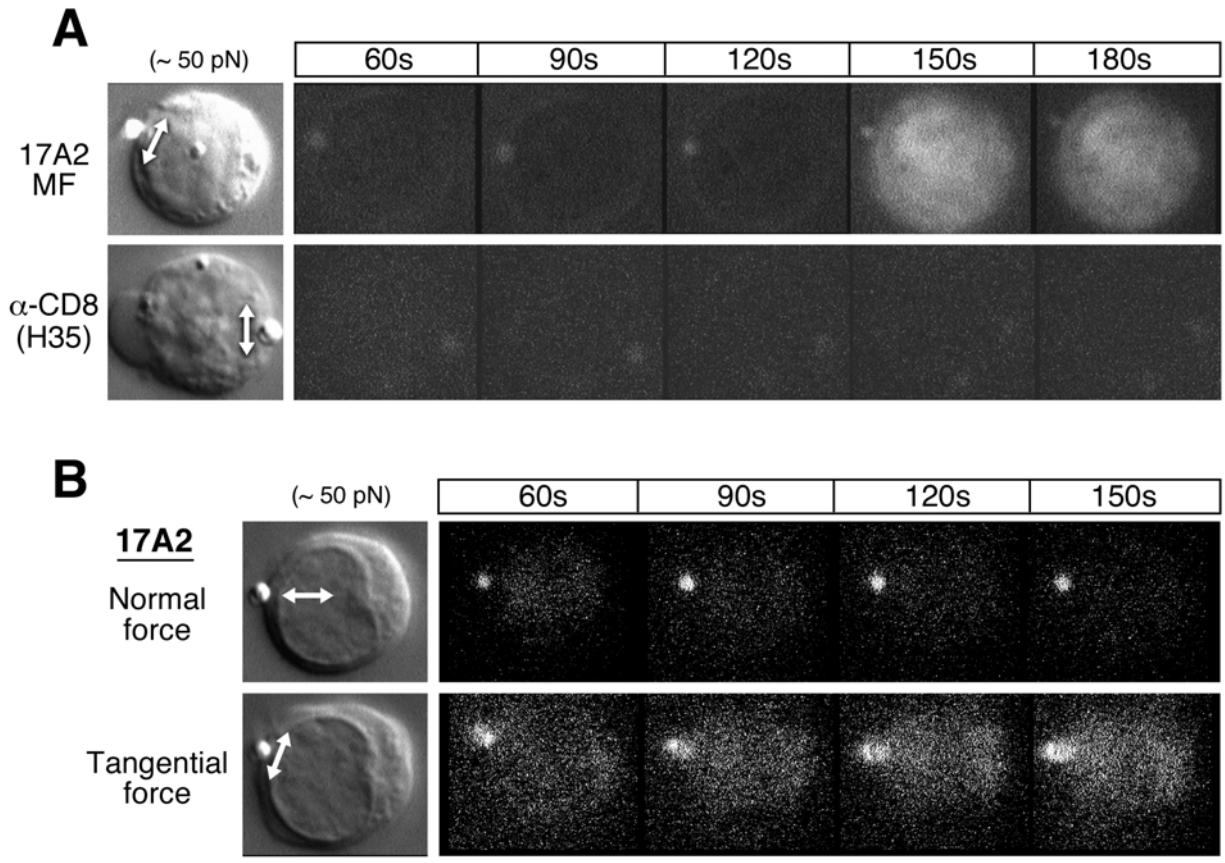

Figure S9. Calcium flux in T cell after application of external mechanical force using optical tweezers

(A) Calcium flux in T cells after application of external tangential mechanical force using optical tweezers. Equivalent amounts of alexa555-labeled 17A2 or H35 (anti-CD8β) was immobilized on protein G-coupled polystyrene beads. (B) Differential forces on 17A2 beads by optical tweezers. T cell-bead contact was manipulated via the trapping beam as shown in bright field images (left panel). The direction of the external mechanical force for a 17A2 bead is denoted by the double-headed arrow. Microscopic fluorescence images were recorded using a 532 nm laser for both alexa555 and calcium orange under temperature control at 37^{o}C. First normal and then tangential force was applied to the same cell. Tangential force is the one acting in the direction of a tangent to the path of the cell body, whereas a normal force acts at right angles to the tangent (see double-headed white arrows). Data are representative of three independent experiments.

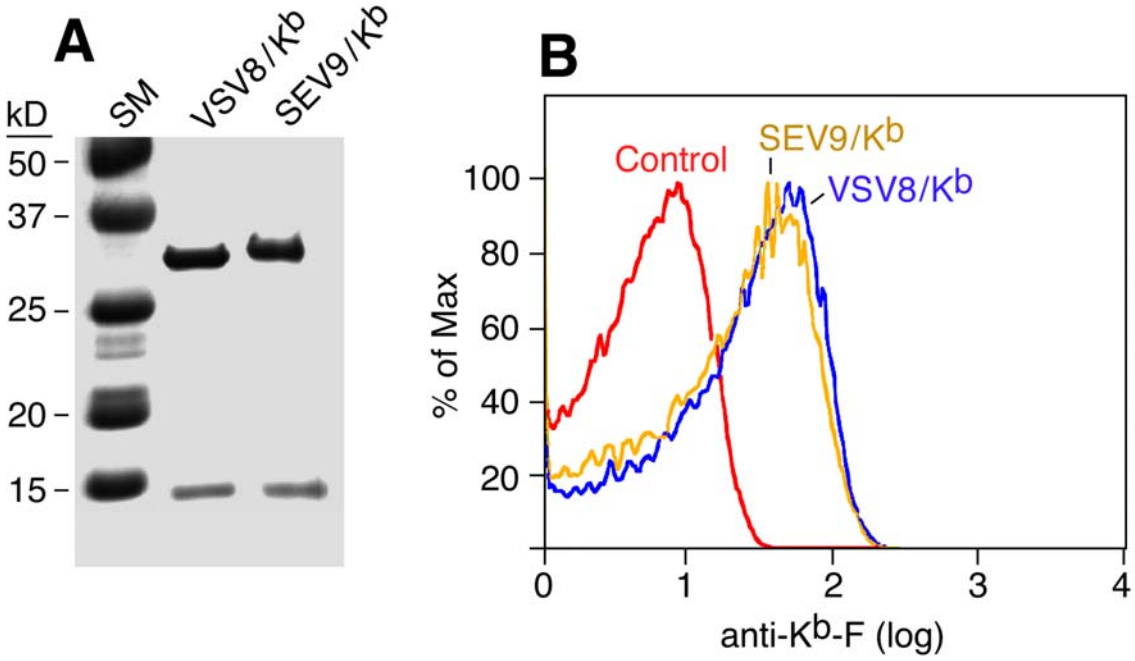

Figure S10. Immobilization of pMHC on beads. (A) SDS-PAGE analysis. Purified H-2K^b complexes after biotinylation and alexa555 labeling were analyzed by gradient gel (8-20%) and then stained by coomassie blue. (B) Flow cytometric analysis of indicated H-2K^b complexes immobilized on beads. 1 μM of each H-2K^b complex was used for coupling to streptavidin polystyrene beads. The coupled H-2K^b complexes were quantitated on beads using flow cytometry and directly FITC-labeled anti-H-2K^b mAb (AF6-88.5) immunofluorescence (anti-K^b-F).

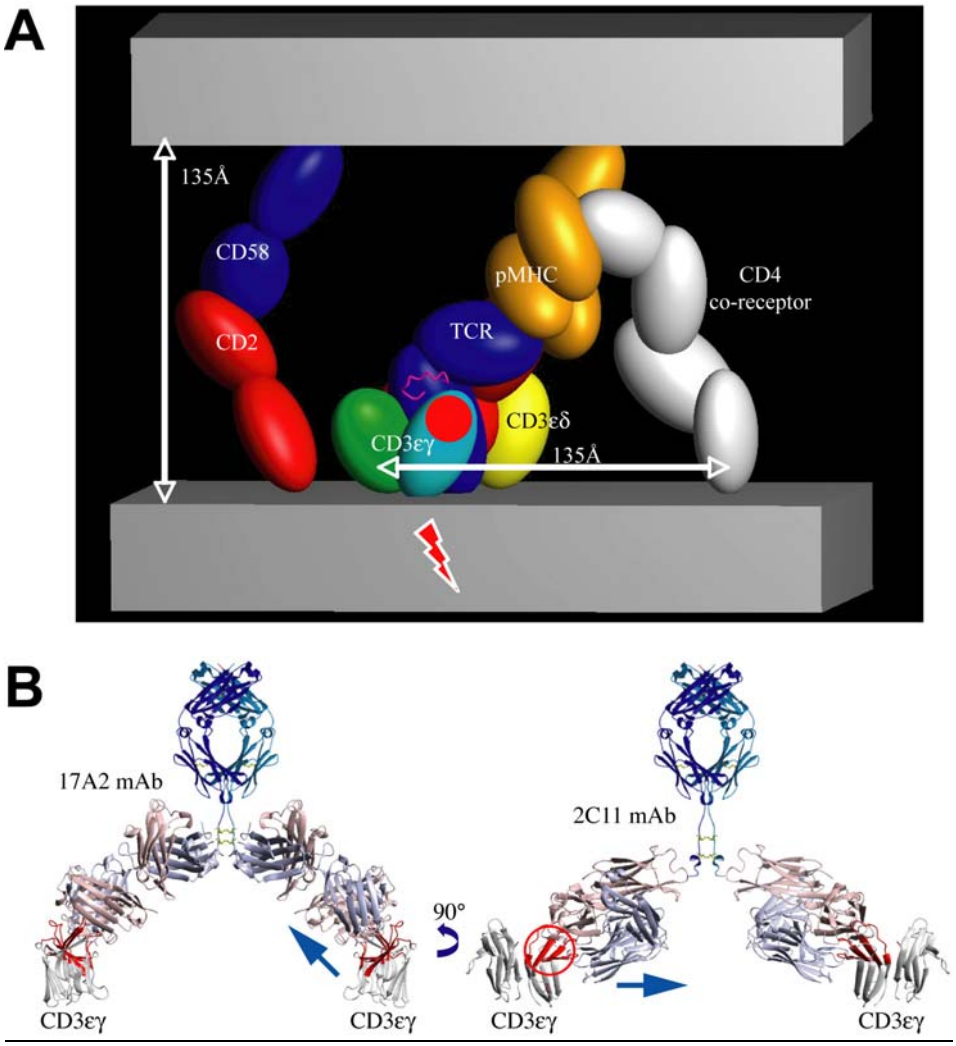

Figure S11. Models illustrating T-cell signaling at the immunological synapse and by specific bivalent anti-CD3 mAbs(A) TCR/pMHC ligation and orientation are optimized by the inter-membrane distances (135 Å) maintained by CD2/CD58 co-receptors at the immunological synapse. The approach of the pMHC toward T-cell surface also favors conjoint interaction with the CD4 co-receptor for optimized kinase cascade signaling. Note the distance between CD3 dimers and CD4 domain 4 is significant, accommodating glycans (not shown). (B) Anti-CD3 mAbs 17A2 and 2C11 bind CD3εγ dimers in orientations orthogonal to each other with 17A2 more upright and 2C11 more tangential. Thus, small perturbations by 2C11 mAb which bound to the TCR complex can result in signaling. In contrast, the 17A2 mAb bound TCR complex, only large external force applied along direction out of plane can exert the necessary torque to trigger signaling. Furthermore, the signaling actions are likely achieved in the same area where the TCRβ FG loop potentially impinging on CD3εγ (filled red circle in panel A) and 2C11 pulling on CD3εγ (unfilled red circle in panel B).