Supplementary Figures for

Mechanical force regulates ligand binding and function of PD-1

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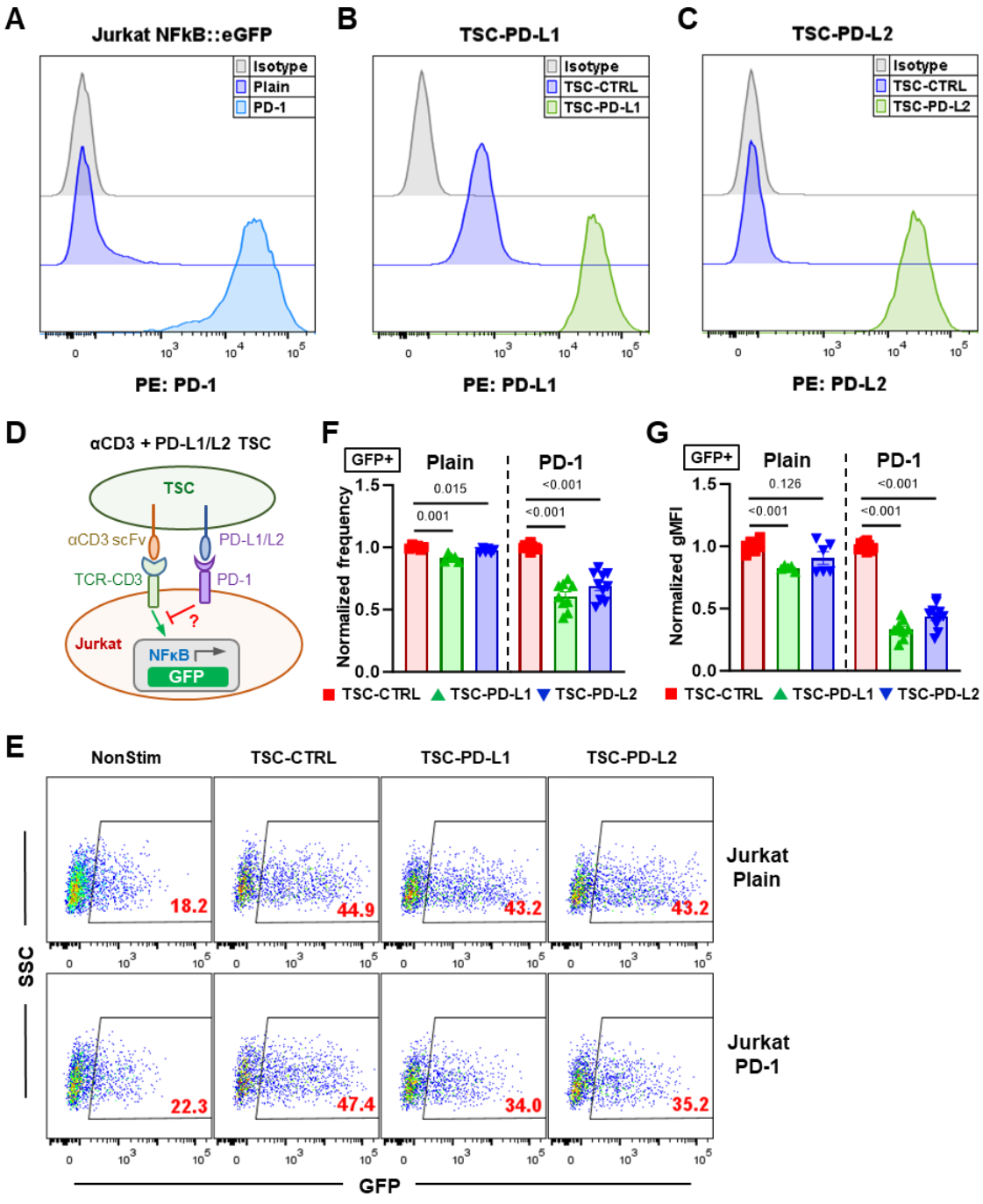

Fig. S1. Detection of PD-1 mediated suppression of TCR-CD3 signaling using NFκB::eGFP reporter Jurkat cells. (A) Flow cytometry histograms of PD-1 staining of plain or PD-1 transduced NFκB::eGFP reporter Jurkat cells. (B-C) Flow cytometry histograms of PD-L1 (B) or PD-L2 (C) staining of TSC without transducing PD-1 ligands (TSC-CTRL) or transduced with PD-L1 (TSC-

PD-L1) or PD-L2 (TSC-PD-L2). (D) Schematics of stimulating NFκB::eGFP reporter Jurkat cells with T-cell stimulator cells (TSC) expressing a single-chain fragment variable (scFv) of anti-CD3 (clone OKT3) and PD-L1 or PD-L2. (E) Representative SSC *vs* GFP plots of reporter Jurkat cells 24 hr after stimulation with indicated conditions. (F-G) Quantification of GFP expression for conditions in B. n = 5 - 6 for plain pooled from 3 independent experiments or n = 9 - 10 for PD-1 reporter cells pooled from 5 independent experiments. Normalized frequency (F) and normalized geometric mean fluorescence intensity (gMFI) (G) were calculated as (sample – averaged background)/(TSC control – averaged background) and presented as mean +/- SEM. Numbers on graphs represent p values calculated from two-tailed student t-test. Source data are provided in Source Data file.

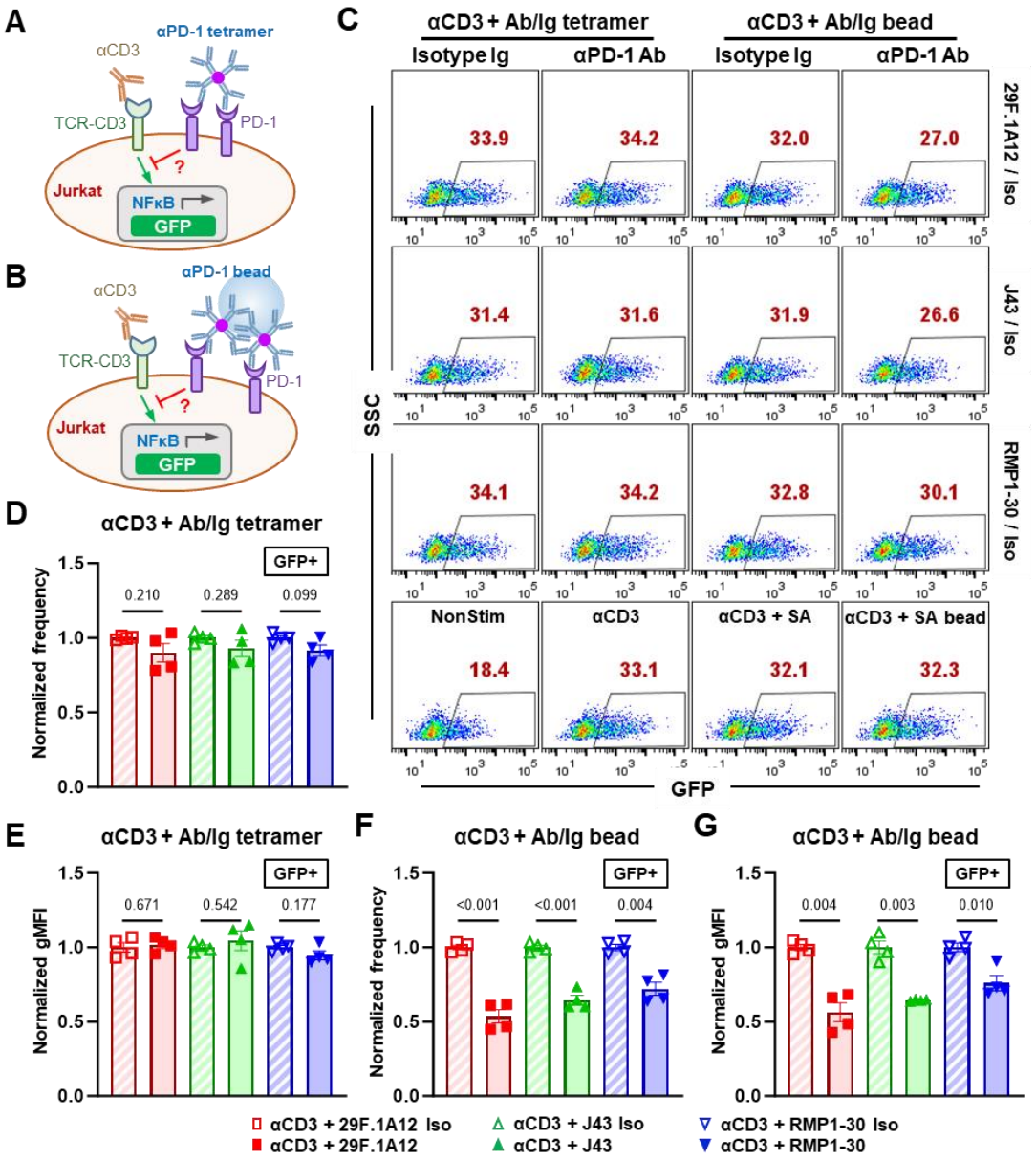

Fig. S2. Immobilized anti-PD-1 antibody triggers PD-1 function. (A&B) Schematics of stimulating NFκB::eGFP reporter Jurkat cells with soluble anti-CD3 and soluble (A) or bead-coated (B) anti-PD-1 antibody (or isotype Ig). (C) Representative SSC *vs* GFP plots of reporter Jurkat cells 24 hr after stimulation with indicated conditions. (D-G) Quantification of GFP expression for conditions in A. n = 4 for all conditions pooled from two independent experiments. Normalized frequency (D&F) and normalized gMFI (E&G) were calculated as (sample – averaged background)/(isotype control

– averaged background) and presented as mean +/- SEM. Numbers on graphs represent p values calculated from two-tailed student t-test. Source data are provided in Source Data file.

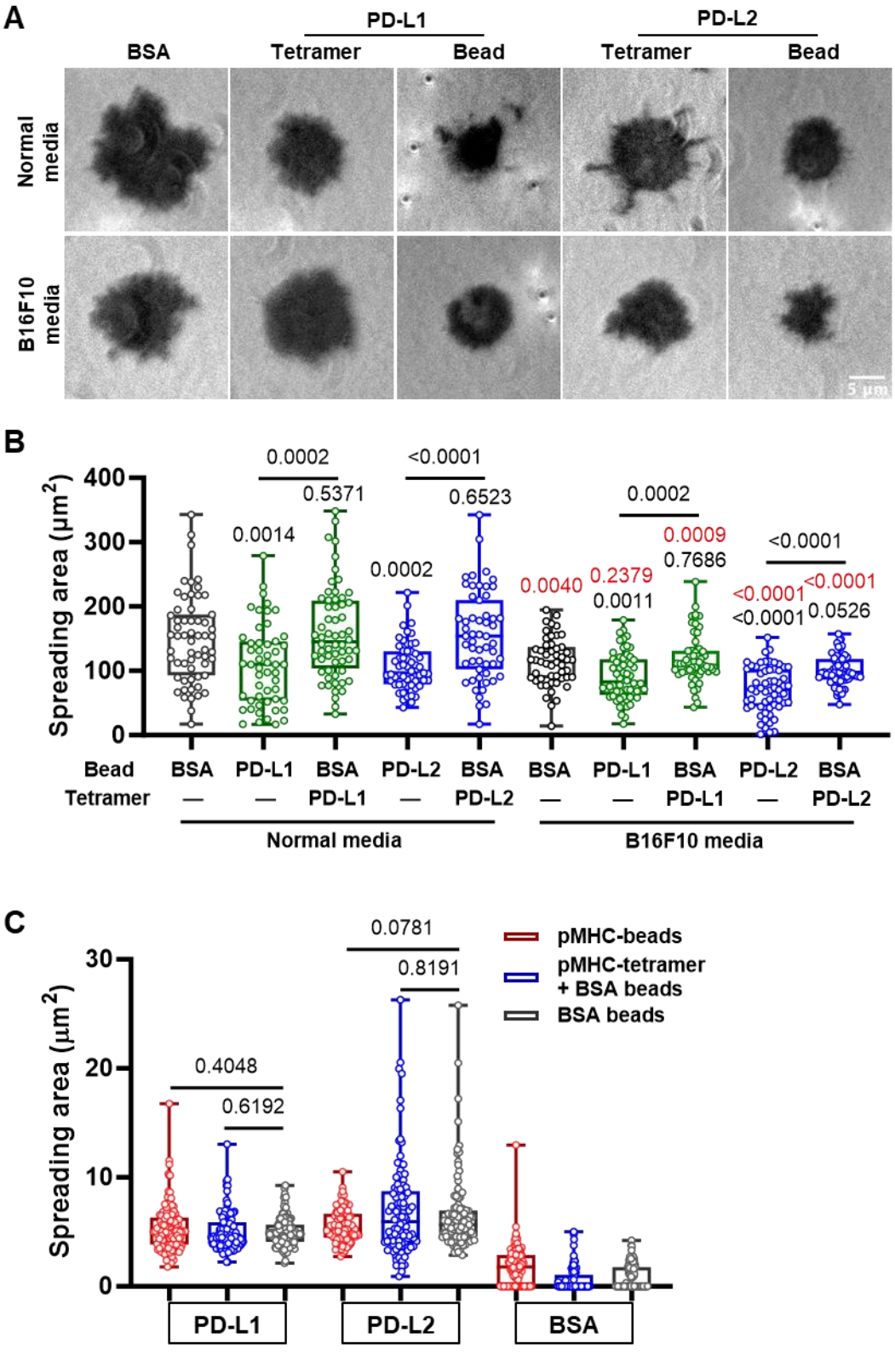
A
PD-L1
PD-L2
BSA
Tetramer
Bead
Tetramer
Bead
Normal media
B16F10 media
5 µm
B
Spreading area (µm²)
400
300
200
100
0
0.0002
<0.0001
0.5371
0.6523
0.0014
0.0002
0.0002
0.0040
0.2379
0.0009
0.7686
<0.0001
<0.0001
<0.0001
0.0011
<0.0001
0.0526
Bead
BSA
PD-L1
BSA
PD-L2
BSA
BSA
PD-L1
BSA
PD-L2
BSA
Tetramer
—
—
PD-L1
—
PD-L2
—
—
PD-L1
—
PD-L2
Normal media
B16F10 media
C
Spreading area (µm²)
30
20
10
0
0.0781
0.8191
0.4048
0.6192
pMHC-beads
pMHC-tetramer
+ BSA beads
BSA beads
PD-L1
PD-L2
BSA

Fig. S3. Respective effects of tumor cell media and TCR stimulation on T cell spreading on pMHC and on PD-Ligands, respectively. Representative RICM images (A) and quantification (B) of activated $CD8^+$ OT1 T cells spreading on SIINFEKL:H2-K^b coated surface in contact with beads coated with PD-L1, PD-L2, or BSA in the presence of tetrameric PD-L1, PD-L2, or BSA in solution. The experiment was done in either normal media (*upper row*, pH = 7.76) or B16F10 melanoma cell-conditioned media (*lower row*, pH = 7.02). n = 59, 52, 60, 57, 55, 51, 60, 57, 60, and 59 cells. Black numbers on graphs represent p values calculated from two-tailed Mann-Whitney U test of indicated two groups or PD-Ligand groups (green or blue) with BSA control (black). Red numbers on graphs represent p values calculated from two-tailed Mann-Whitney U test of normal media vs B16F10 media for each group. (C) Engagement of TCR with bead-bound or tetrameric pMHC in solution had no effect on T cell spreading on PD-L1/L2-coated surface. Activated OT1 T cells spreading on PD-L1, PD-L2, or BSA surface in contact with SIINFEKL:H2-K^b or BSA coated beads in the absence of presence of tetrameric SIINFEKL:H2-K^b in solution. n = 119, 115, 121, 109, 105, 111, 110, 102, and 106 cells from 1 of 2 independent experiments. Data were presented in box (median with 25%/75% boundaries) and whisker (min and max) plots. Numbers on graphs represent p values calculated from two-tailed Mann-Whitney U test of indicated two groups or the experiment group (green or blue) with BSA control (black). Source data are provided in Source Data file.

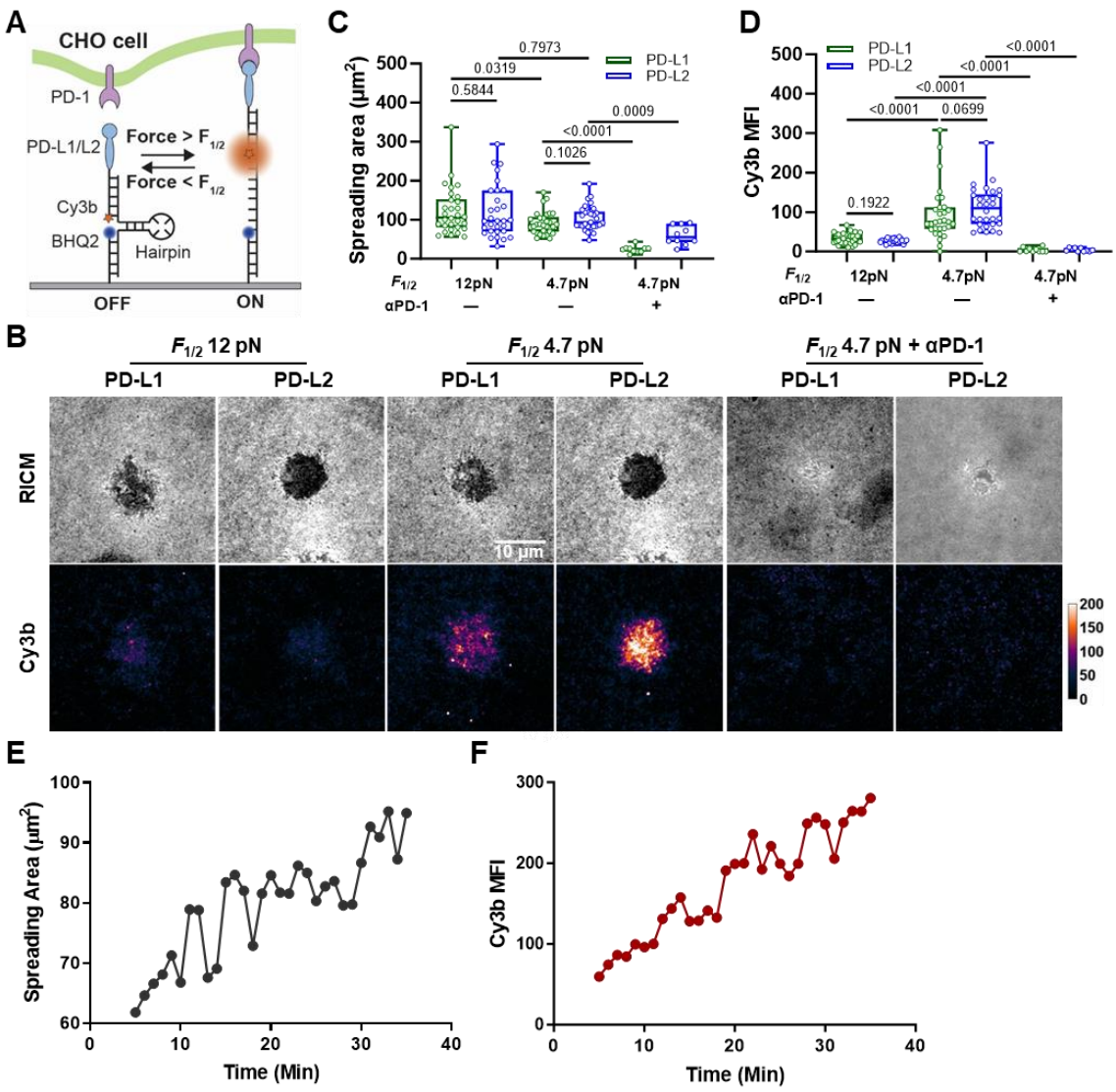

Fig. S4. DNA-based MTPs reveal active cellular forces applied to PD-1–PD-Ligand bonds. (A) Schematics of detecting cellular forces on PD-1–PD-Ligand bonds using DNA-based molecular tension probes (MTPs). Forces above threshold unfold the hairpin, which separates Cy3B from the BHQ2 to de-quench the fluorophore. (B) Representative reflection interference contrast microscopy (RICM) and total internal reflection fluorescence (TIRF) images of PD-1 expressing CHO cells 30 min after landing on glass surface functionalized with MTPs of indicated conditions. For PD-1 blockade, cells were pre-incubated with PD-1 blocking antibody clone 29F.1A12 before imaging. (C-D) Quantification of cell spreading area (C) and tension signal (D) for conditions in B. n = 30,

30, 30, 30, 10, and 10 pooled from 3 independent experiments. Data were presented in box (median with 25%/75% boundaries) and whisker (min and max) plots. Numbers on graphs represent p values calculated from two-tailed Mann-Whitney U test. (E&F) Realtime quantification of spreading area (E) and Cy3b fluorescence (F) of a PD-1 CHO cell spreading on PD-L2-coupled MTP of 4.7 pN threshold force as shown in Video S2. Source data are provided in Source Data file.

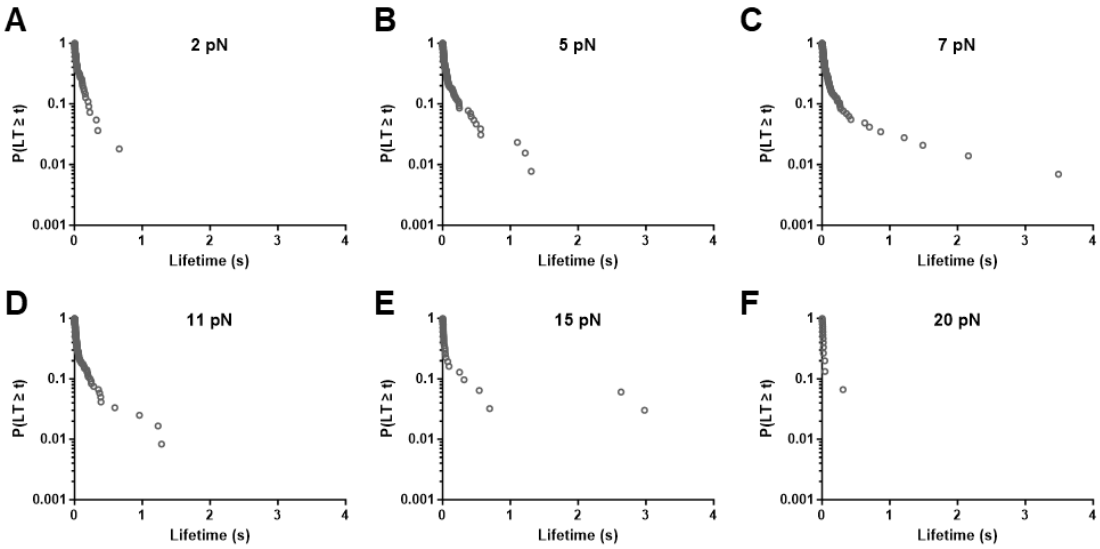

Fig. S5. Survival frequencies (fraction of event with lifetime greater than *t*) of PD-1–PD-L1 bond lifetime for different force bins. n = 55, 129, 144, 120, 33, and 16 lifetime events for (A) to (F) respectively. Source data are provided in Source Data file.

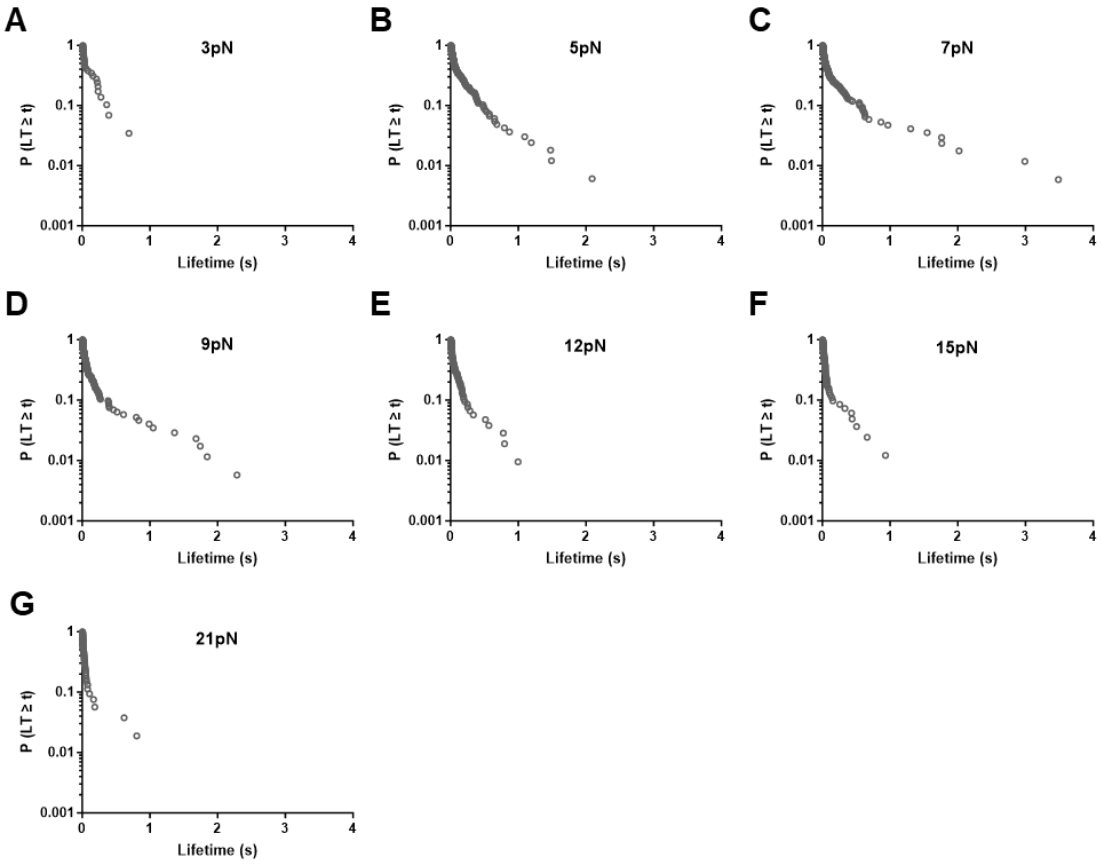

Fig. S6. Survival frequencies (fraction of event with lifetime greater than *t*) of PD-1–PD-L2 bond lifetime for different force bins. n = 29, 165, 170, 173, 105, 82, and 53 lifetime events for (A) to (G) respectively. Source data are provided in Source Data file.

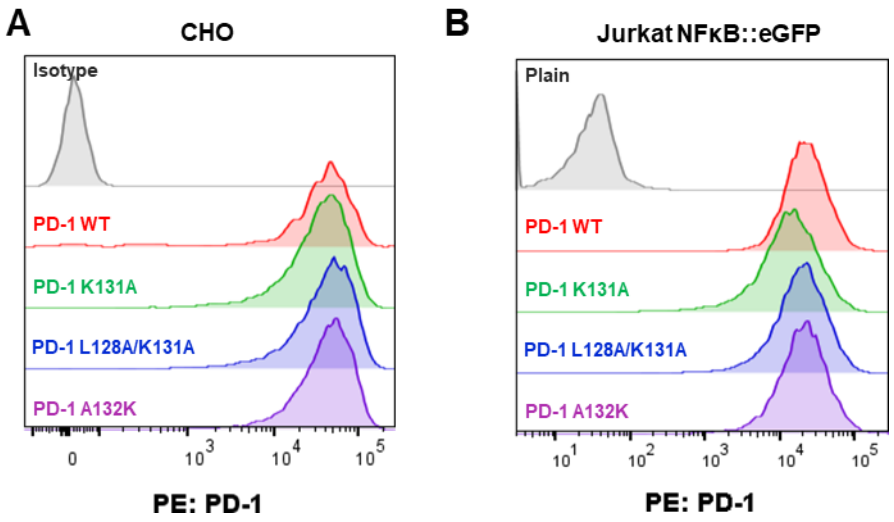

Fig. S7. Flow cytometry histograms comparing PD-1 staining of CHO cells (A) or NFκB::eGFP reporter Jurkat cells (B) expressing WT or indicated mutants of PD-1.

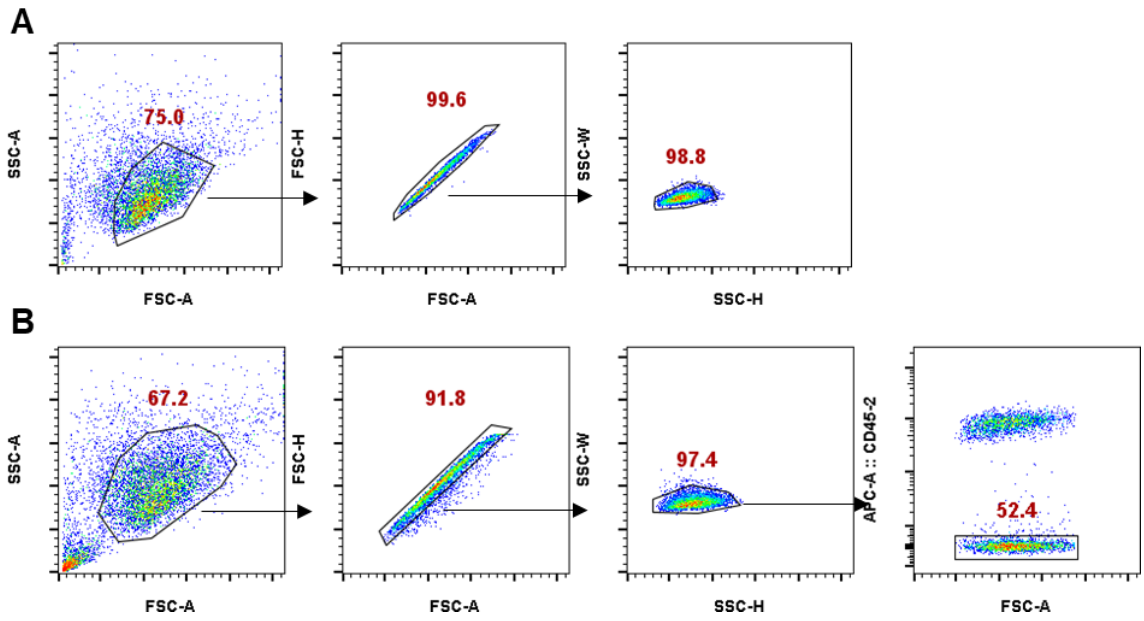

Fig. S8. Gating strategies for flow cytometry data. (A) Sequential gating for data with one cell population to quantify GFP induction, or expression of surface PD-1, PD-L1, or PD-L2 (related to Figs. 1, S1A-C, S2, and S7). (B) Sequential gating or reporter Jurkat cells co-cultured with CD45.2+ TSC cells (related to Figs. 7, and S1E-G).