Supplementary Information for

Lck bound to coreceptor is less active than free Lck

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The establishment of endogenous Lck knockout OT-I hybridoma cell model

The OT-I TCR$\alpha\beta$ recognizes the chicken ovalbumin peptide $OVA_{257-264}$ (SIINFEKL) presented by H2-K^b (1), and a T hybridoma cell line expressing OT-I TCR has been used extensively in studies of TCR and co-receptor interactions (2-4). We previously used OT-I hybridoma cells overexpressing Lck-fluorescent fusion proteins to investigate coreceptor-bound or -unbound Lck functions (4), but these cells have a low yet detectable amount of endogenous Lck expression (**Figure S1C,D**), which can exist in either free or coreceptor-bound Lck pools. Endogenous Lck may be preferred over the exogenous Lck molecules during signal transduction, that could lead to incorrect interpretations of differences between the overexpressed free and coreceptor-bound Lck constructs. The existence of endogenous Lck may also complicate further analysis of the recruitment of Lck to the IS. To eliminate these concerns, we established an endogenous Lck knockout OT-I hybridoma system using CRISPR/Cas9 (5). The single guide RNA (sgRNA) of endogenous Lck was designed to target an intron-exon junction of Lck genomic DNA (gDNA), to prevent the Cas9 from cutting the exogenous Lck cDNA (**Figure S1A**). The CRISPR/Cas9 system was delivered into an OT-I.CD8$\alpha\beta^{-/-}$ cell line together with CD8$\alpha\beta$ coreceptor using the pBMN-Z retroviral system (**Figure S1B**). Cell clones that expressed both CD8α and CD8β were selected and confirmed to have a knockout of endogenous Lck using Western Blotting (WB), flow cytometry, and DNA sequencing (**Figure S1C-E**). The endogenous Lck knockout did not affect other markers expressed on the hybridoma cells (**Figure S1E**). This Cas9 expressing hybridoma cell line is a convenient tool for future studies on other gene

knockouts. As a control, a Lck-sufficient hybridoma cell line over-expressing the Cas9-CD8α and CD8β was also developed (OT-I.CD8$\alpha\beta^+$.Cas9$^+$). Loss of endogenous Lck in the hybridoma cells (endoLck$^{-/-}$ OT-I hybridoma cells) abolished TCR signaling, as Erk was not phosphorylated after TCR activation with pMHC (**Figure S1F**). TCR signaling in these hybridoma cells was restored by overexpressing Lck-mCherry (wild-type Lck), Lck(C20.23A)-mCherry (free Lck; Lck mutant that cannot bind to CD4 or CD8), or CD8αLck-mCherry (Lck constitutively bound to CD8α) (**Figures S1C-F**). Notably, Erk phosphorylation was enhanced in the free Lck-expressing hybridoma cells, compared to the WT Lck or CD8-bound Lck-expressing cells (**Figure S1F**). The Erk signaling strength differences may be due to the different roles of free and coreceptor bound Lck in TCR signaling. As expression of either form of Lck rescued Erk signaling after TCR stimulation, this experimental system is suitable for studying the roles of different Lck pools in early TCR signaling events.

Material and Methods

Mice

Wild Type (WT) C57BL/6J and OT-I transgenic mice on the same background were used. All mice used in this research were at the age of 5 to 8 weeks and were bred at NUS Comparative Medicine animal facility, under approved IACUC protocol BR17-0478. Mice were age and sex matched in each experiment.

Cell lines and cell culture methods

RPMI, IMDM and DMEM medium, Fetal Bovine Serum (FBS), HEPES and PBS were from HyClone. F-12 media, L-Glutamine (L-Glu), non-essential amino acid (NEAA), Sodium pyruvate and Blasticidin were purchased from Gibco. Penicillin and Streptomycin (Pen-Strep) were bought from Thermo Fisher Scientific. β-mercaptoethanol (β-ME) was bought from Sigma-Aldrich. Primary mouse T cells were cultured in complete RPMI (cRPMI) medium: 10% FBS, 100 U/ml penicillin-100 μg/ml streptomycin, 2 mM L-Glu, 5 μM β-ME, 0.11 mg/ml sodium pyruvate, 10 mM HEPES and NEAA. OT-I Cytotoxic T cells (CTLs) were generated from OT-I transgenic mice splenocytes. Red blood cells (RBCs) were lysed with ACK lysis buffer and the rest splenocytes were cultured in cRPMI supplemented with 100 U/ml recombinant human IL-2 (rhIL-2) (R&D) and 10 nM OVA peptide for 24-48 hours, followed by resting with only IL-2 for CTLs or with 10 ng/ml IL-15 (R&D) for memory T cell differentiation. HEK293T and Platinum-E (Plat-E) cell lines were cultured with complete DMEM (cDMEM): 10% FBS, 2 mM L-Glu, 100 U/ml penicillin-100 μg/ml streptomycin and NEAA. OT-I T hybridoma cells were cultured with complete IMDM medium: 10% FBS, 2 mM L-Glu, 100 U/ml penicillin-100 μg/ml streptomycin, 5 μM β-ME, 500 μg/ml G418 (selecting for TCRα) and 3 μg/ml puromycin (selecting for TCRβ).

Retroviral virus gene delivery of Lck mutants

pBMN-Z vectors containing Lck-eGFP, Lck(C20.23A)-mCherry, CD8α-Lck-Cerulean and CD3ζ-eGFP were previously described (4). All restriction enzymes were bought from New England BioLabs. Lck(Y394F)-mCherry, Lck(Y505F)-mCherry and Lck(K273R)-mCherry mutants were made using the

Quickchange Site Mutagenesis Kit (Stratagene). One Shot Stbl3 competent cells (1730102; Invitrogen) were used for pBMN-Z transformation. Plat-E retroviral packaging cell line was used for producing retrovirus for transduction. The transductions were performed as previously described (4). Transduced cells were sorted based on fluorescence using MOFLO XDP Sorter (Beckman Coulter) at NUS Immunology Programme Flow Core Facility.

Lck sgRNA design and cloning

The Lck sgRNA candidates were selected from the CHOPCHOP website (6). For screening Lck sgRNAs that guide the Cas9 protein only to edit the endogenous Lck but not the exogenous Lck and Lck mutants, Lck sgRNAs that targeting intron and exon junction were selected based on the off-targets and efficiency ranking provided on the CHOPCHOP website. The Lck sgRNAs were cloned by overlap PCR into TOPO vectors and then sent for sequencing before cloning into pBMNz-Lck(C20.23A)-mCherry vectors. The sequence of selected Lck sgRNA: TGTGGTGCAGGAGCGGTGAGTGG. U6-sgRNA was cloned into pBMNZ-CD8β plasmid using SalI and NotI restriction sites. The pBMNz-CD8β-U6-sgRNA plasmid was used for CRISPR knock out of OT-I hybridoma cells.

Liposomes and lipid bilayer preparation

1,2-dioleoyl-sn-glycero-3-phos-phocholine (DOPC) was mixed with 0.2 mol% 1,2-dioleoyl-sn-glycero-3- [(N-(5-amino-1-carboxypentyl) iminodiacetic acid)succinyl] (Ni-NTA-DOGS) and 0.2 mol% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-Cap-Biotinyl (CAP-biotin-PE) in chloroform (all the lipids components were bought from Avanti Polar Lipids, Inc). The chloroform

was evaporated by N_2 followed by drying with speedvac for 2.5 hours. After chloroform was completely removed from the lipid cake, the liposomes were dissolved in deoxygenized deionized water on ice overnight followed by sonication until the liposome solution became transparent. The liposome solution was then filtered through a 0.22-mm filter. The final liposome is 4 M DOPC and CAP-Biotin-PE. The lipids were further deoxygenized and kept in N_2 atmosphere at 4°C. Lab-TeK chambers used for lipid bilayer formation were treated with 6 M NaOH for 2 hours and rinsed with deionized water extensively. NaOH treated chambers were stored in deionized water until the experiments. Chambers were dried and added a 10-fold-diluted lipid suspension (0.4 mM final). After 30 minutes incubation, each well was rinsed with PBS extensively. Lipid bilayers were then blocked (5 mg/ml BSA in PBS) and incubated sequentially with 5 μg/ml streptavidin for 30 minutes followed by 1 μg/ml his-ICAM (4) in blocking buffer for 30 minutes. After loading proteins, the bilayers were rinsed and warmed before adding the cells.

Spinning disk confocal microscopy and FRAP analysis

OT-I hybridoma cells transduced with Lck(C20.23A)-mCherry or CD8αLck-Cerulean were added to the lipid bilayers supplemented only with Streptavidin-APC (Invitrogen) and his-ICAM, not MHC. Cells were cultured at 37 °C for 30 minutes before the imaging, allowing the cells to adhere to the lipid bilayers. FRAP experiments were performed on a Zeiss spinning disk confocal microscope. Cells were kept in a humidified incubation chamber at 37°C with 5% CO_2 during image collection. Images were analyzed using ImageJ (NIH). FRAP measurements were full-scale normalized according to a previously

described method (7). The resulting data were fit to a single exponential curve model, and the mobile fraction and half-time recovery was calculated (8, 9).

Sodium orthovanadate preparation and pervanadate stimulation

Sodium orthovanadate solution (180 mM) were prepared by firstly dissolving 3.68 g Na_3VO_4 (MW = 183.91 g/mol) into 100 ml dH2O. The solution was then adjusted to pH = 10, followed by boiling until clear and colorless. The solution was cooled down until room temperature on ice, and the pH of the solution were increased at this point. The pH adjustment, boiling and cooling down steps were repeated until the pH of the solution stabilized at around 10. The final volume of the solution was around 110 ml and the final concentration of activated Na_3VO_4 solution was 180 mM. The Sodium orthovanadate solution was then aliquoted and stored at -20°C. Pervanadate stock solution (30 mM) was made by sequentially adding 772.3 μl of PBS, 166.7 μl of Sodium orthovanadate (180 mM) followed by 61 μl of 3% H_2O_2. The solution turned a little appearance of yellow color. The mixture was incubated for 15 minutes at room temperature in the dark, before adding to the cells. The pervanadate stock solution can be stored at room temperature up to 24 hours. The stock solution was then diluted as required for different experiments. As a positive control for phosphorylated Lck, OT-I hybridoma cells were stimulated with 100 mM pervanadate for 5 minutes at 37°C and the stimulation was quenched by adding ice cold PBS.

T cell stimulation by H-2K^b OVA APLs tetramer, plate-bound anti-CD3ε and anti-CD28 antibodies and anti-CD3 crosslinking.

H2-K^b-OVA APLs monomers (NIH Tetramer Core Facility) were tetramerized according to the NIH Tetramer Core Facility protocol. 5-10 x 10^6 naïve CD4 T cells, naïve CD8 T cells, OT-I CTLs or OT-I hybridoma cells were pelleted and resuspended in 200 μl cRPMI. Cells were vortexed and pre-warmed in 37**°**C for 30 seconds and supplemented with 800 μl pre-warmed cRPMI with or without H2-K^b-OVA APLs tetramers. To quench the stimulation, 2 ml ice cold PBS was added into cells to quench the stimulation. Naïve CD4 and CD8 T cells, OT-I hybridoma cells were stimulated with plate bound antibodies. 96-well plate or 6-well plate were pre-coated with 1 μg/ml anti-mouse CD3ε (clone 145-2C11, invitrigen) and 1μg/ml anti-mouse CD28 (clone 37.51, invitrogen) antibodies at 4°C overnight. Cells were added into the antibody pre-coated plates for the indicated stimulation time. Stimulated or unstimulated T cells were used for FC-IP, sequential IP or imaging experiments. For the anti-CD3ε crosslinking stimulation method, 0.5 million hybridoma cells were harvested for each stimulation condition. Hybridoma cells incubated with 2μg anti-mouse CD3ε-biotin (clone eBio500A2, eBioscience) antibody on ice for 30 minutes, followed by adding 1mg/ml streptavidin (Life technologies) and immediately incubate in 37 °C water bath for indicated stimulation time. Stimulation were stopped by adding 2 ml ice cold PBS. Cells were then centrifuged and lysed.

Immunoprecipitation (IP) and Western Blotting (WB)

Double positive (DP) T cells, naïve CD4 and naïve CD8 T lymphocytes were freshly isolated from B6 mice or OT-I transgenic mice, followed by sorting based on $CD4^+$ $CD8^+$, $CD4^+$ $CD44^{lo}$ or $CD8^+$ $CD44^{lo}$, respectively. T cells (15 x 10^6) were lysed in Brij97 lysis buffer (150 mM NaCl, 50 mM HEPES, 1% Brij97,

protease-phosphatase inhibitor cocktail (Roche) and 20 μM PP2 inhibitor (Sigma)) on ice for 20 minutes. 30-40 μl of whole cell lysate (WCL) was kept as input. Protein G beads (Invitrogen) were coated with anti-CD4 (clone GK1.5, eBioscience) or CD8α (clone 53-6.7, BD Pharmingen) antibody followed by crosslinking by BS^3 (Invitrogen; 21580). The crosslinking can remove the heavy and light chains during the WB, as Lck's molecular weight (56 kDa) is similar to heavy chain (~ 55 kDa). Then the crosslinked protein G beads were mixed with WCLs and incubated at 4°C with rotation for at least 2 hours. For the sequential IP, 40 million DP cells and 20 million naïve CD4 or CD8 T cells were lysed with 400 μl and 250 μl lysis buffer. 80μl of CD4 or CD8α antibody crosslinked Dynabeads were used for each round of Immunoprecipitation. 40 μl of WCL or supernatants were kept after each IP. Additional 40 μl of WCl and supernatant 3 were kept for FC-IP analysis. For DP sample, first IP was split into CD4 and CD8α IP. The second and third IP were performed with a mix of 80 μl CD4 coated and 80 μl CD8α crosslinked Dynabeads. For each Western Blotting, 12 μl of each sample was loaded into each well. OT-I hybridoma cells were lysed with NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, protease-phosphatase inhibitor cocktail and 20 μM PP2 inhibitor). All samples were mixed with SDS sample buffer (Nacalai Tesque Inc.) and boiled for 5 minutes at 95°C prior to loading onto the SDS-Page gel or storage at -80°C. 12% SDS-PAGE gel (Invitrogen) was used for Lck detection, for a better separation of Lck and Fyn. 4%-12% SDS-PAGE gel (Invitrogen) were used for hybridoma samples. PVDF-FL membrane was bought from Millipore. The following antibodies were used for Western Blotting detection: anti-Lck (clone 3A5) was purchased from Santa Cruz Biotechnology. Anti-pY416 Src (D49G4),

anti-pY505-Lck, anti-Non-P-Src(Y416) (7G9), anti-Erk1/2, anti-pErk1/2, anti-PLC-γ, anti-Zap70, anti-pY319-Zap70, anti-Lat, anti-pY191-Lat anti-Fyn, anti-β-actin and anti-GAPDH antibodies were purchased from Cell Singaling Technology. Anti-Flag (clone M2) antibody was purchased from Sigma. Anti-pY142-CD3ζ antibody is bought from BD Biosciences, anti-CD3ζ antibody was bought from Thermo Scientific. Secondary antibodies goat anti-rabbit IgG, IRDye 680LT-conjugated and goat anti-mouse IgG, IRDye 800-conjugated and blocking buffer were bought from Li-Cor Biosciences.

Flow Cytometry-Immunoprecipitation (FC-IP)

Flow cytometry immunoprecipitation (FC-IP) was done as described previously (10). Briefly, anti-CD4 (clone RM 4-4, BD Pharmingen), anti-CD8β (clone eBioH35-17.2, eBiosience), anti-CD8α (clone 53-6.7, BD Pharmingen) anti H-2Kb (AF6-88.5, BioLegend) was coated onto CML beads (Invitrogen, C37255). Ab coated beads were used to pull-down CD4, CD8 or H-2Kb. Beads were then washed and split equally for different staining regimes. PE-conjugated anti-CD4 (clone GK1.5, BD Pharmingen), anti-CD8α (clone 53-6.7, BD Pharmingen), anti-CD8β (clone BioH35-17.2, eBioscience) anti-Lck (clone 3A5, Santa Cruz) antibodies were used to quantify Lck/CD8 and Lck/CD4 coupling ratios. The PE conjugated antibodies were purified with Superdex 200 10/300 GL size exclusion column for an Ab:PE 1:1 ratio. For 24 hours T cell stimulation experiments, the unstimulated cells were directly washed with PBS and pellet down before freezing in the -80°C freezer. The same number of stimulated cells were also washed and pelleted down and freezing in the -80°C freezer for at least overnight. The unstimulated and stimulated cells were lysed and

underwent FC-IP together during analysis. BV711-anti-CD4 (RM4-4, BD Biosciences), APC-anti-H-2Kb (AF6-88.5.5.3, eBioscience), APC-anti-CD4 (GK1.5, BD Biosciences), BUV395-anti-CD8β (eBioH35-17.2, BD Biosciences), APC-anti-CD8α (53-6.7, invitrogen) antibodies were further involved for testing if the capture and detection antibody clones may interfere the staining of each other. Calibration beads (BD Quantibrite beads, 340495) were analyzed along with each experiment. MFI of each staining was calculated into PE-molecules per bead using the calibration equation calculated by the Quantibrite bead (**Figure S5**). The percentage of Lck bound to coreceptors were calculated by calculated Lck/bead on CD4 (or CD8β) IP beads subtracting background (anti-Lck signal /bead on H-2Kb IP beads) devided by the CD4(or CD8α)/bead on CD4 (or CD8β) IP beads subtracting background (anti-CD4 or CD8α signal/bead on H-2Kb IP beads).

Flow Cytometry

The following antibodies are used for Flow Cytometry staining: APC-anti CD4 (GK1.5), PE(or APC)-anti-CD8α (53-6.7), BUV395-anti-CD8β (eBioH35-17.2) BUV395-anti-CD25 (PC61), BV421-anti-CD44 (IM7), PE-anti-CD69 (H1.2F3), FITC-anti-CD62L (MEL-14), APC-anti-Vα2 (B20.1), PE-anti-Vβ5 (MR9-4). Antibodies used for Flow Cytometry staining were purchased from eBioscience, Biolegend, and BD Biosciences. Surface staining was performed by incubating live cells with antibodies for 30 minutes on ice. For intracellular staining, cells were fixed with IC Fixation buffer (eBioscience, 00-8222) and permeabilized with 1 X Permeabilization buffer (from 10x stock, eBioscience, 00-8333). Flow cytometry was carried out with BD LSRFortessa X-20 (Becton Dickinson). DP,

SP, naïve T cells were sorted by Sony Cell Sorter SY3200 (Synergy) and hybridoma cells were sorted with MOFLO XDP Sorter (Beckman Coulter). Data were analyzed using FlowJo software (TreeStar).

Lck kinase activity assay

The Lck kinase activity assay was performed as previously described (11), with some modifications. 5 million hybridoma cells were lysed with 500 ul NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 3X protease-phosphatase inhibitor cocktail) and different Lck-mCherry fusion proteins were immunoprecipitated using 2 μg anti-mCherry antibody (Clone 16D7, Invitrogen) and 80 μl Dynabeads (Invitrogen) overnight . The harvested beads were washed three times in kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM ATP) and incubated for 20 min at 37 °C in 50 μl of kinase buffer containing 500 ng recombinant human CD3ζ-GST (Sino Biological). The reaction was quenched by adding 6x SDS sample buffer (Nacalai Tesque Inc.) at 95 °C followed by incubating at 95 °C for 3 min. Lck activity was measures though the anti-pY142-ζ (Cat. 558402, BD Biosciences) intensity using the near-infrared WB.

Mouse IL-2 ELISA

OT-I hybridoma cells were stimulated plate-bound 1μg/ml anti-CD3e (eBioscience) for 24 hours. After stimulation, the cells were pelleted down, and supernatants were collected directly for ELISA analysis or kept at -20 °C until needed. IL-2 ELISA experiments were done according to the manufacturer's protocol, Mouse IL-2 Uncoated ELISA Kit (Thermo Fisher Scientific). The

optical densities of samples were measured using Varioskan Flash (Thermo Fisher Scientific).

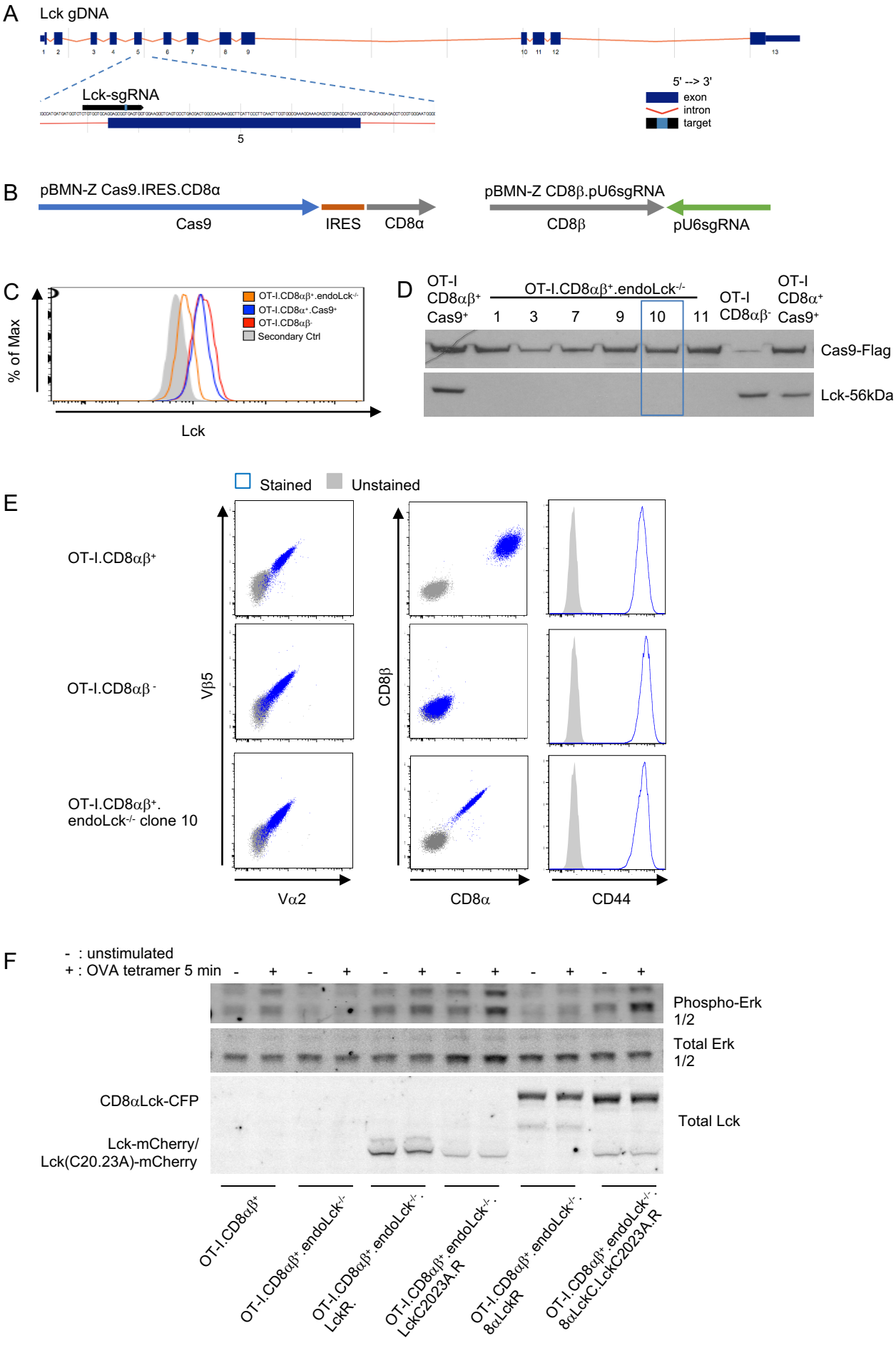
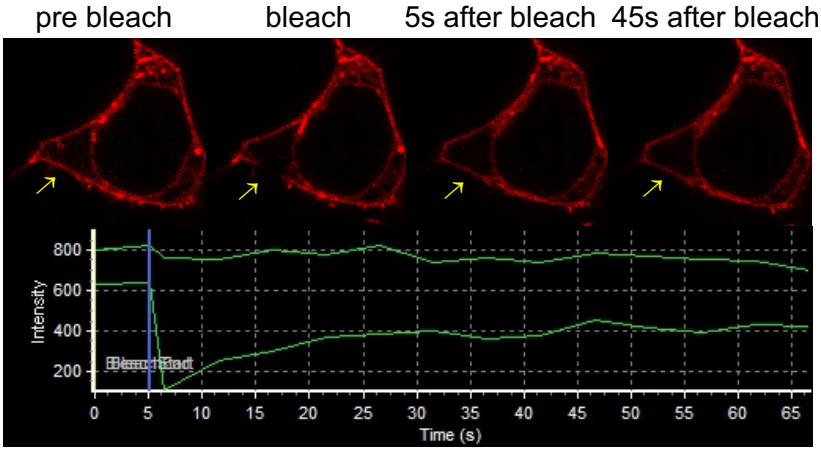

A
Lck gDNA
Lck-sgRNA
5' --> 3'
exon
intron
target
B
pBMN-Z Cas9.IRES.CD8α
Cas9
IRES
CD8α
pBMN-Z CD8β.pU6sgRNA
CD8β
pU6sgRNA
C
% of Max
OT-I.CD8αβ+.endoLck-/-
OT-I.CD8α+.Cas9+
OT-I.CD8αβ-
Secondary Ctrl
Lck
D
OT-I CD8αβ+ Cas9+
OT-I.CD8αβ+.endoLck-/-
1 3 7 9 10 11
OT-I CD8αβ-
OT-I CD8α+ Cas9+
Cas9-Flag
Lck-56kDa
E
Stained
Unstained
OT-I.CD8αβ+
OT-I.CD8αβ-
OT-I.CD8αβ+. endoLck-/- clone 10
Vβ5
Vα2
CD8β
CD8α
CD44
F
- : unstimulated
+ : OVA tetramer 5 min
- + - + - + - + - + - +
Phospho-Erk 1/2
Total Erk 1/2
CD8αLck-CFP
Lck-mCherry/ Lck(C20.23A)-mCherry
Total Lck
OT-I.CD8αβ+
OT-I.CD8αβ+.endoLck-/-
OT-I.CD8αβ+.endoLck-/- LckR.
OT-I.CD8αβ+.endoLck-/- LckC2023A.R
OT-I.CD8αβ+.endoLck-/- 8αLckR
OT-I.CD8αβ+.endoLck-/- 8αLckC.LckC2023A.R

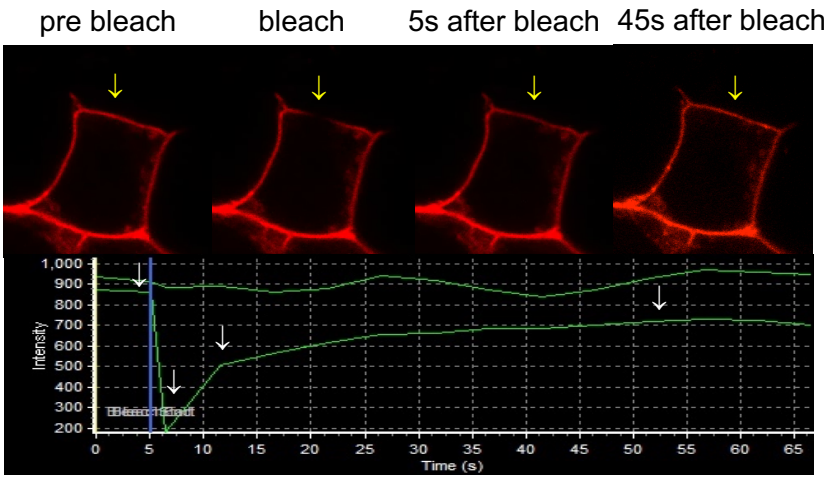
Figure S1: The establishment of endogenous Lck knocked out OT-I hybridoma cell line by using CRISPR/Cas9 system.

(A) The sgRNA used for endogenous Lck knock out targets the intron-exon5 junction of Lck gDNA. (B) pBMN-Z Cas9.IRES.CD8α and pBMN-Z CD8β.pU6sgRNA5 were transduced into OT-I.CD8αβ^{-} cells. (C) OT-I hybridoma cells before and after transduction with pBMN-Z Cas9.IRES.CD8α and pBMN-Z CD8β.pU6sgRNA5 were stained with anti-Lck antibody and the Lck expression levels were detected by flow cytometry. (D) The Flag-Cas9 and Lck expression levels were detected and compared by WB between the Lck knockout clones and control cells (OT-I CD8αβ^{+} Cas9^{+}). Clone 10 was selected for further experiments. (E) The surface expression of TCR (Vα2 and Vβ5), coreceptors (CD8α and CD8β) and effector-memory T cell marker CD44 on hybridoma cells were measured by flow cytometry. (F) The OT-I.CD8αβ^{+}.endoLck$^{-/-}$ cells transduced with different Lck mutants (Lck-mCherry, LckC20.23A-mCherry, and/or CD8αLck-mCherry) were stimulated (+) by H2-K^{b}-OVA tetramers for 5 minutes or not stimulated (-). The Erk phosphorylation levels of each cell line were detected by WB. The figure represents at least two independent experiments.

A HEK293T cells over-expressing CD8α-Lck-mCherry



B HEK293T cells over-expressing Lck(C20.23A)-mCherry



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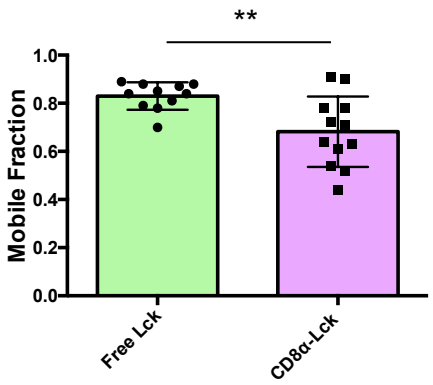

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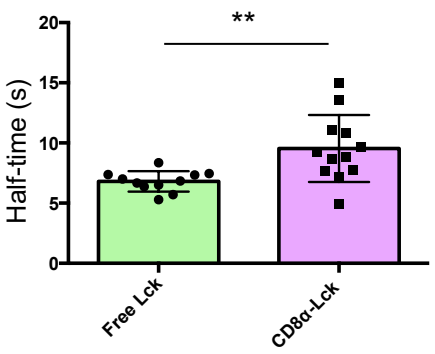
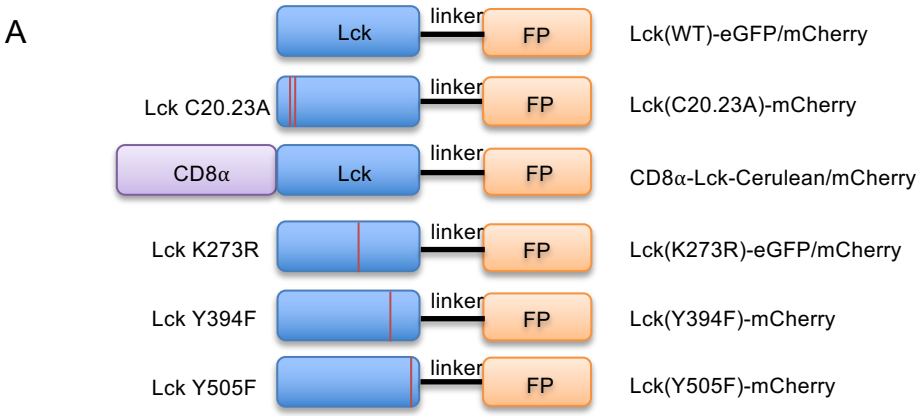
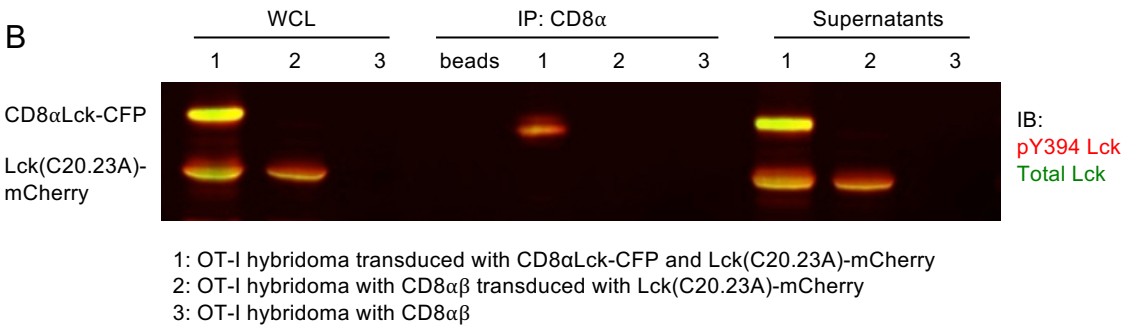


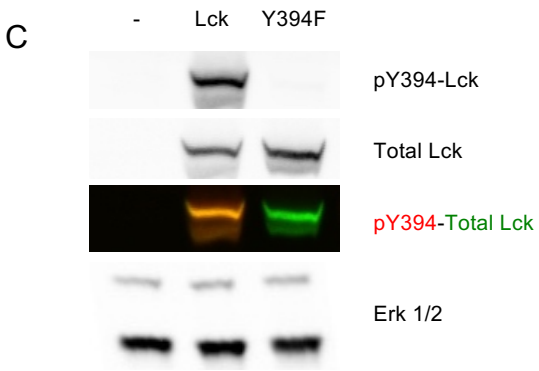
Figure S2: FRAP imaging of free Lck and CD8α-Lck in 293T cells.

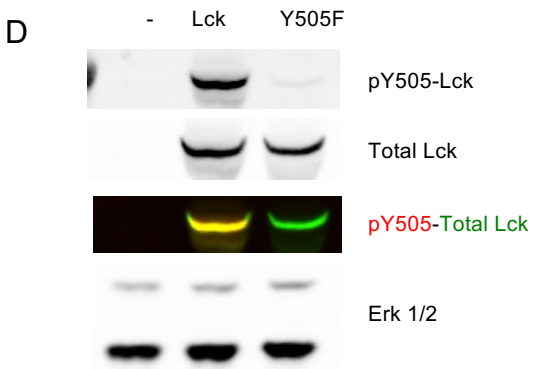
HEK293T cells were transfected with either free or CD8α-bound Lck that was fused with mCherry. The cells then underwent FRAP imaging and analysis. Images were acquired by confocal microscope. (A) The images and the intensity recovery curve of CD8α-bound Lck. The intensity recovery curve of the photo bleached area is pointed by white arrows. The intensity curve of the control area is above the intensity curve of the photobleached area. (B) The images and the intensity recovery curve of free Lck. The photo-bleaching location is marked by the yellow arrows. Images acquired at: pre-bleach, first image, 5 s and 45 s after bleach and the corresponding timepoints are shown by the white arrows on the fluorescent intensity recovery curve below. (C) & (D) The mobile fraction and half-time (s) were analyzed by Prism software. The significance was analyzed by student's t test, ** $p<0.01$. Mean ± s.e.m of Lck(20.23A)-mCherry n=11 and CD8α-Lck-mCherry n=12 is presented. The figure is a representative of two independent experiments.





1: OT-I hybridoma transduced with CD8αLck-CFP and Lck(C20.23A)-mCherry
2: OT-I hybridoma with CD8αβ transduced with Lck(C20.23A)-mCherry
3: OT-I hybridoma with CD8αβ





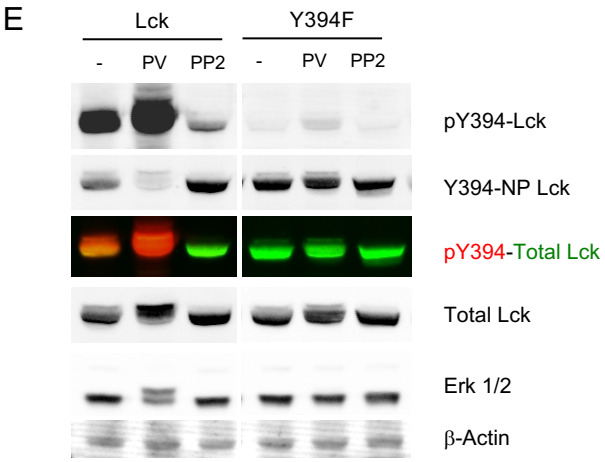
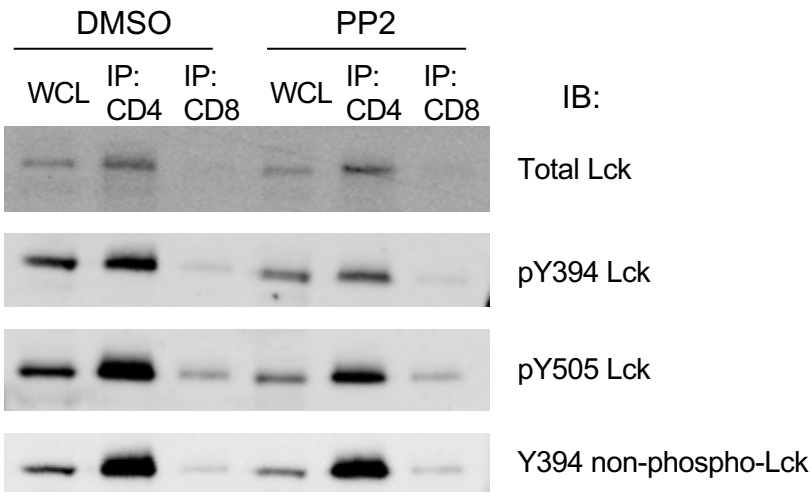


Figure S3: Fluorescent protein (FP) linked Lck constructs and anti-Lck antibody verification.

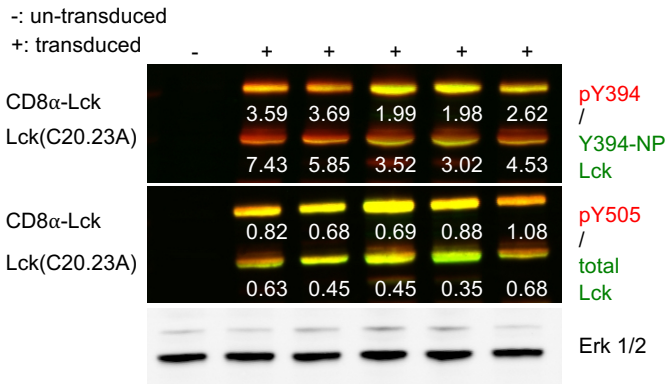
(A) The list of FP linked Lck constructs (WT and mutants) that were involved in this research. FPs linked to Lck constructs are eGFP, mCherry or Cerulean. (B) OT-I hybridoma cells were transduced with Lck(C20.23A)-mCherry and/or CD8αLck-Cerulean constructs. The cells were lysed and IP with anti-CD8α antibody. The pY394 (red) and total (green) Lck proteins were blotted. The yellow color indicates both pY394 and total Lck were presented at the same location. The "Supernatant" is the cell lysis harvested after the IP incubation. Anti-pY416-Src antibody was used to test pY394-Lck. OT-I Hybridoma cells were not transduced (-), transduced with Lck-mCherry, Lck(Y394F)-mCherry (C) or Lck(Y505F)-mCherry (D). The hybridoma cells were lysed and analyzed by WB. Anti-total Lck(3A5), anti-pY394 Lck (anti-pY416 Src) anti-pY505 Lck and total Erk1/2 antibodies were used for the detection. Total Erk was used as a loading control. (E) OT-I hybridoma cells overexpressing Lck-mCherry or Lck(Y394F)-mCherry were unstimulated, stimulated with Pervanadate (PV) for 5 minutes or treated with PP2 inhibitor for 1 hour before lysis. Signal intensity ratio of pY394/Y394-NP Lck were calculated.

A

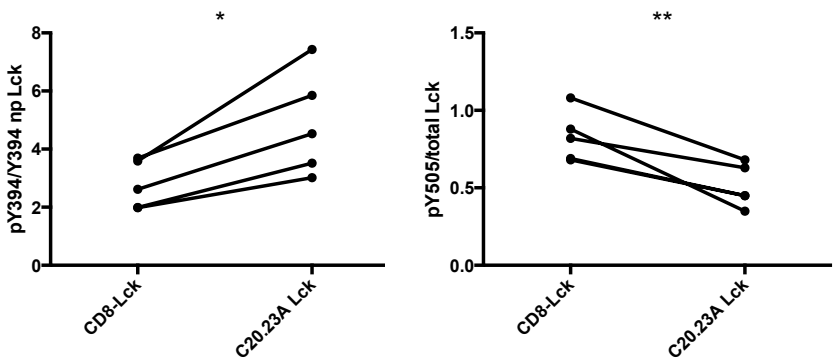


DMSO: Same volume of DMSO was added as negative controls
PP2: 20μM PP2 was added into lysis buffer

B



C



D

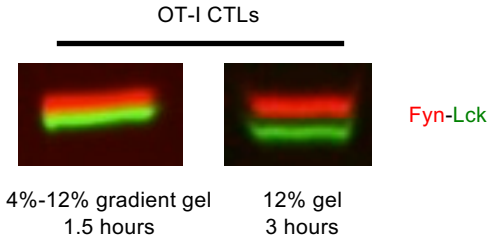
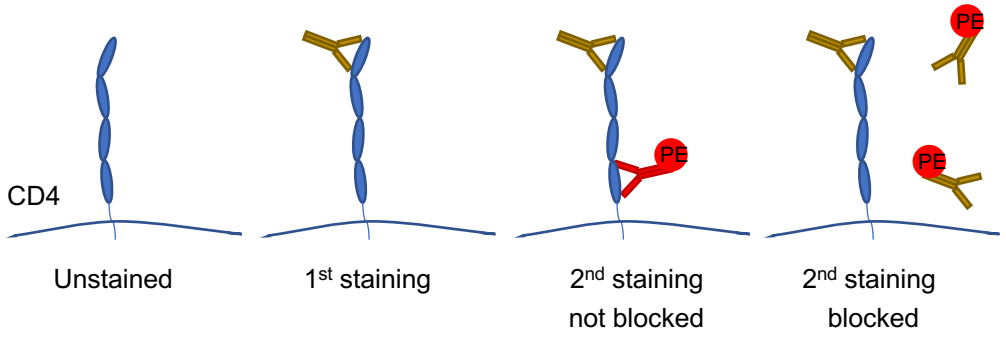


Figure S4: Free Lck fraction was more Y394 phosphorylated than CD8-bound Lck fraction in OT-I hybridoma cells.

(A) Mouse DP cells were sorted and lysed in Brij97 lysis buffer with 20mM PP2 inhibitor or the same volume of DMSO as controls. The cell lysates were IP with anti-CD4 or anti-CD8α antibodies. Total pY394, pY505 and Y394-non-phosphorylated-Lck were detected. One representative of two independent experiments is shown. (B) OT-I hybridoma cells transduced with both CD8α-Lck-Cerulean and Lck(C20.23A)-mCherry were lysed. Five technical repeats are presented. The anti-pY394 and Y394-non-phospho Lck were blotted on the same membrane, so as pY505 and total Lck antibodies. (C) The intensity ratio of pY394/Y394-NP Lck or pY505/total Lck is presented. The intensity ratios were statistically analyzed, and the significance was calculated by paired t-test, * $p<0.05$ and ** $p<0.01$. One representative of three independent experiments is shown. (D) WB optimization for Lck and Fyn separation for the sequential IP. OT-I CTLs were lysed and cell lysates were loaded onto gradient gel and 12% constant gel. The protein electrophoresis was running at 150V for 1.5 hours and 3 hours respectively.

A


CD4
PE
PE
PE
Unstained
1st staining
2nd staining
not blocked
2nd staining
blocked

B

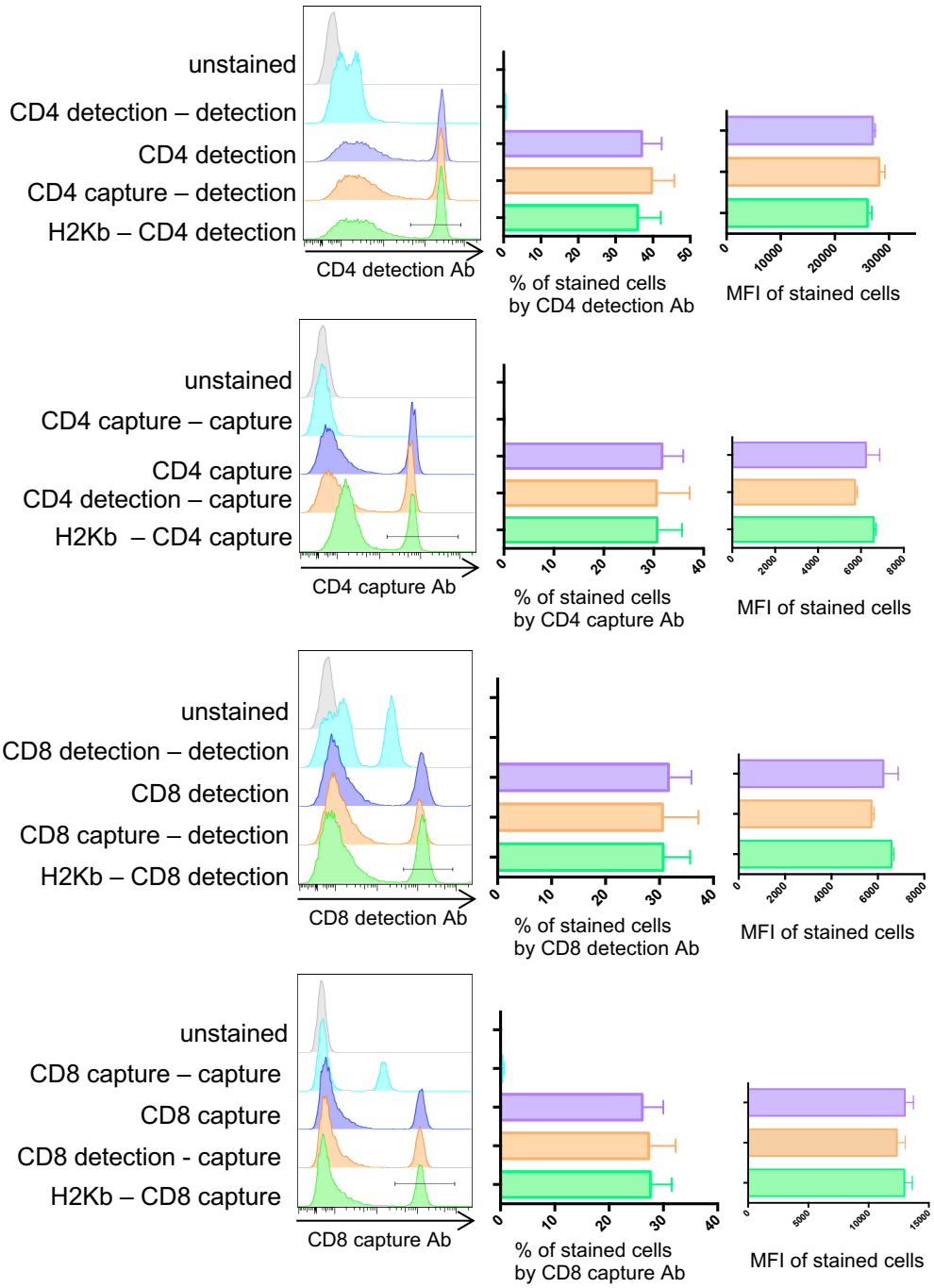

unstained
CD4 detection – detection
CD4 detection
CD4 capture – detection
H2Kb – CD4 detection
CD4 detection Ab
% of stained cells
by CD4 detection Ab
MFI of stained cells
unstained
CD4 capture – capture
CD4 capture
CD4 detection – capture
H2Kb – CD4 capture
CD4 capture Ab
% of stained cells
by CD4 capture Ab
MFI of stained cells
unstained
CD8 detection – detection
CD8 detection
CD8 capture – detection
H2Kb – CD8 detection
CD8 detection Ab
% of stained cells
by CD8 detection Ab
MFI of stained cells
unstained
CD8 capture – capture
CD8 capture
CD8 detection - capture
H2Kb – CD8 capture
CD8 capture Ab
% of stained cells
by CD8 capture Ab
MFI of stained cells

Figure S5:

CD4 and CD8 capture and detection antibodies do not block each other.

(A) Diagram illustrates the design of the experiment. Coreceptor CD4 is used as an example. Cells harvested were either unstained, or stained with a unconjugated capture or detection antibody, followed by a second staining with the capture or detection antibody. The second staining may be blocked when the antibodies recognize the same epitope of the molecule; or not blocked when antibodies recognize different epitopes of the molecule or different molecules. (B) Primary mouse lymphocytes were harvested from WT mice, 2 million cells were used for each staining. Each sample is performed with either single antibody staining with wash, or followed by a second staining with a different antibody (different clone or different fluorescent tag). H2Kb staining followed by CD4 detection served as no blocking control. CD4 detection antibody staining followed by detection antibody staining served as maximum blocking control, The first staining is either using antibodies with no fluorescence tag or using antibodies has a different fluorescent tag. The histogram, percentage of stained cells and MFI of CD4 detection antibody were compared with the CD4 capture followed by detection antibody staining, and H-2Kb followed by CD4 detection antibody staining. CD4 capture, CD8 detection/capture antibodies were tested with the same strategy. The figure is a representative of two independent experiments.

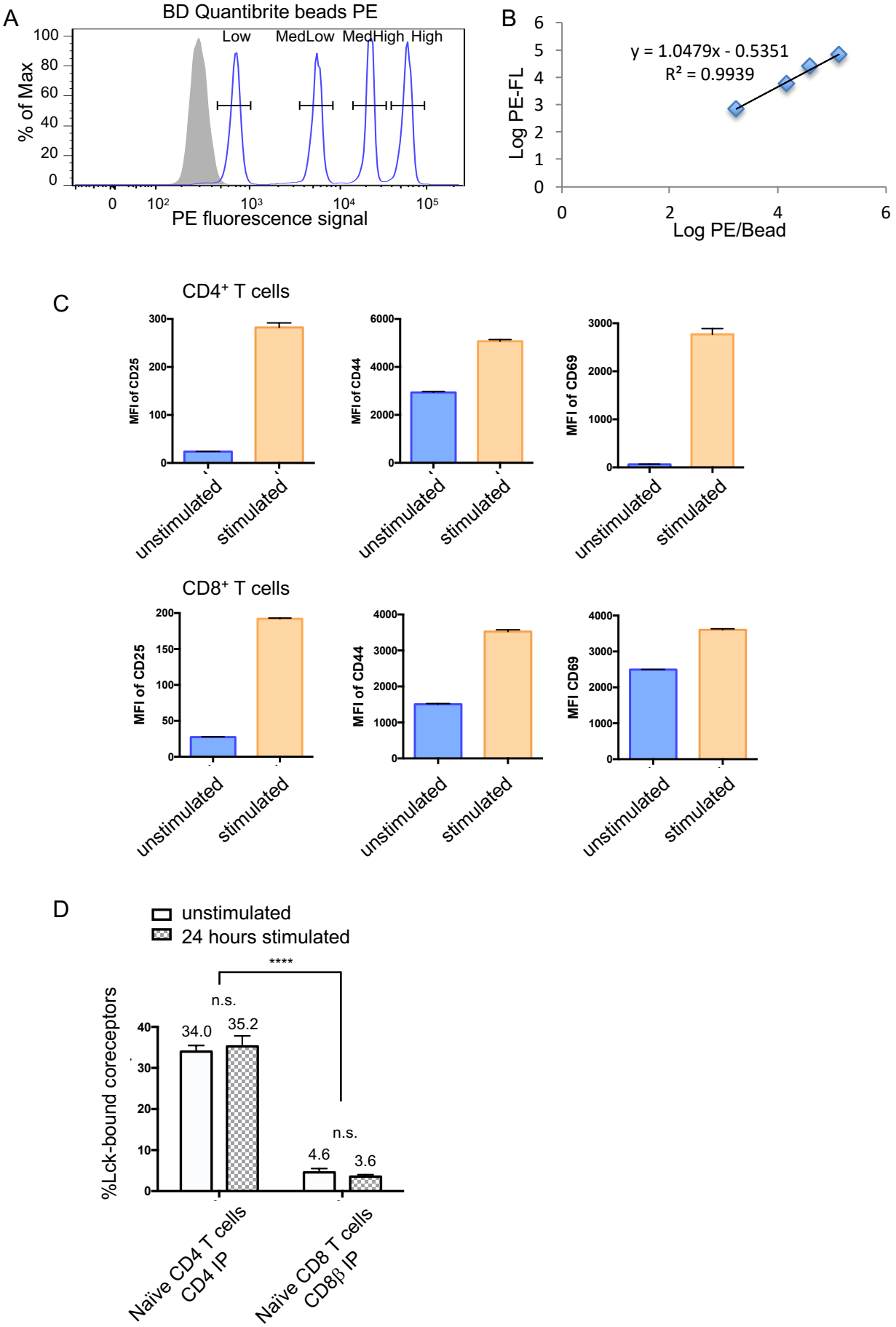

A
BD Quantibrite beads PE
Low
MedLow
MedHigh
High
% of Max
PE fluorescence signal
B
y = 1.0479x - 0.5351
R² = 0.9939
Log PE-FL
Log PE/Bead
C
CD4⁺ T cells
MFI of CD25
MFI of CD44
MFI of CD69
unstimulated
stimulated
CD8⁺ T cells
MFI of CD25
MFI of CD44
MFI CD69
unstimulated
stimulated
D
unstimulated
24 hours stimulated
****
n.s.
34.0
35.2
n.s.
4.6
3.6
%Lck-bound coreceptors
Naïve CD4 T cells
CD4 IP
Naïve CD8 T cells
CD8β IP

Figure S6: Quantification of coreceptors (CD4 or CD8αβ) and Lck coupling after TCR stimulation.

(A) One example of the flow analysis of BD Quantibrite beads PE and (B) the standard curve generated based on the Quantibrite beads are presented. The MFI of each FC-IP sample were calculated into PE per bead using similar equation generated by the BD Quantibrite beads PE sample run along with the experiment. (C, D) Naïve $CD4^+$ and $CD8^+$ T cells were pre-sorted stimulated (or not stimulated) by plate bound anti-CD3ε and anti-CD28 antibodies for 24 hours. (C) Cell stimulation were analyzed by CD25, CD44 and CD69 staining by flow cytometry. (D) Unstimulated cells were directly frozen after sorting and stimulated cells underwent one freeze-thaw cycle. Both unstimulated and stimulated cells were lysed and proceed with FC-IP analysis together. The percentage of Lck-bound coreceptors in naïve and stimulated $CD4^+$ and $CD8^+$ T cells are presented. Mean ± s.e.m is shown. Data represents three to four independent experiments.

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