Supporting Information

Zhong et al. 10.1073/pnas.1221609110

SI Methods

Cell Lines and General Reagents. Platinum-E retrovirus producing and B16/A2–K^b tumor cell line was cultured in DMEM supplemented with 10% FBS, Glutamax-I, sodium pyruvate, nonessential amino acids, and penicillin–streptomycin. Splenocytes were harvested from B16/A2–K^b transgenic mice and cultured in RPMI medium containing 30 IU/mL human IL-2. For CD8+ splenocytes, the cells were subjected to CD8 negative selection (mouse CD8a+ T Cell Isolation Kit; Miltenyi Biotec) according to manufacturer's instructions. For CD8− splenocytes, the cells were stained with anti-mouse CD8 antibody (eBioscience), and the unlabeled cells (CD8−) were separated and collected by cell sorting [MoFlo Cell Sorter; New York University (NYU) Flow Cytometry Core]. CD8+ or CD8− cells were stimulated on 24-well plates precoated with anti-CD3ε (1 μg/mL) and anti-CD28 (2 μg/mL) antibodies (both from BD Biosciences) for 2 d before retroviral transduction. More than 90% of the stimulated and expanded cells were T cells (1). gp209-2M peptide (IMDQVPFSV) was purchased from Biosynthesis Inc. Antigen-presenting cell (APC) anti-mouse T-cell receptor β (TCR-β) constant (clone H57-597) wand PE anti-mouse CD3ε (clone 145-2C11) were from eBioscience; phycoerythrin (PE) anti-human TCR Vβ8 (clone JR2) was from Biolegend; PE anti-human TCR Vβ14 (clone CAS1.1.3), PE anti-human TCR Vβ17 (clone E17.5F3.15.13), and PE anti-human TCR Vβ13 (clone CAS1.1.3) were from Beckman Coulter. FBS was from Thermo Scientific HyClone, and all other cell culture reagents were from Invitrogen. Human recombinant IL-2 (Aldesleukin Proleukin) was from Novartis.

Retroviral Transduction. Six million retroviral packaging cells platinum-E (Cell Biolabs) were plated overnight in 10-cm poly(lysine) precoated plates (BD Bioscience). Retroviral particles were generated by transfecting the platinum-E cells with the transfection complex formed by Lipofectamine 2000 (Invitrogen), TCR retroviral plasmid, and pcl–Eco helper plasmid (Imgenex). After 2 d, fresh virus supernatant (1 mL) was harvested and mixed with 1×10^6 cells and 10 μg/mL protamine sulfate (APP Pharmaceuticals) in a 24-well plate and centrifuged for 90 min at $2{,}000 \times g$ at 32 °C. The transduced splenocytes were harvested after 5- to 7-d expansion in RPMI medium containing 30 IU/mL human IL-2 (Proleukin).

Intracellular Staining of c-Myc Epitope-Tagged TCRs. A c-Myc tag was inserted in the 3′ end of the TCR constructs by PCR. Splenocytes expressing c-Myc epitope-tagged TCRs were fixed/permeabilized with Cytofix/Cytoperm (BD Biosciences) as per manufacturer's instructions, stained with mouse anti-cMyc antibody (GeneScript), and washed three times, followed by staining with a secondary Alexa 488 anti-mouse IgG antibody (Invitrogen).

Cytotoxicity Assays. B16/A2–K^b cells were loaded with 10 μM gp209 peptide for 2 h and ^{51}Cr (Perkin-Elmer) for 1 h at 37 °C. The cells were washed three times in RPMI medium and resuspended at 3×10^4 cells/mL. Then, 100 μL of cells was mixed with 100 μL of splenocytes transduced with gp209-specific TCRs at indicated ratios. Two controls were included on the same plate: spontaneous release, in which no T cells were added, and maximal release, in which B16/A2–K^b cells were lysed with 2% Triton-X. The plates were incubated for 5 h at 37 °C and 5% CO_2. After incubation, the plates were centrifuged at $311 \times g$ for 5 min. Then, 100 μL of supernatant from each well was mixed with 100 μL of OptiPhase Supermix (Perkin-Elmer) in a 96-well flat-bottom plate and recorded in a MicroBeta2 microplate counter (Perkin-Elmer). Percentage of specific killing is calculated based on the following formula:

$$(\text{experimental release cpm} - \text{spontaneous release cpm}) / (\text{maximal release cpm} - \text{maximal release cpm}) \times 100,$$

where cpm is counts per minute.

Fluorescent Imaging. T2/A2–K^b cells were pulsed with 10 μM gp209–2M peptide for 2 h at 37 °C and washed twice in ice-cold imaging buffer (RPMI medium without Phenol Red) with 0.2% fresh sodium azide. Splenocytes transduced with gp209-specific TCRs were stained with Fura-2 calcium indicator dye (20 μg/mL; Invitrogen) for 15 min at room temperature and washed twice in imaging buffer. T2/A2–K^b cells were kept on ice, and T cells were kept at room temperature in the dark before imaging. The imaging experiments were done on a Zeiss Axiovert 3D-lapse microscope using a 40× Fluar (NA 1.3) objective controlled by Metamorph (Molecular Devices) with a XL-3-TIRF Incubator (PECON) kept at 37 °C and 5% CO^2. T2/A2–K^b and T cells were added to an imaging chamber (Lab-Tek) coated with poly(L-lysine) solution (Sigma). Only T cells captured before conjugate formation and forming stable conjugate for at least 5 min are included in the analysis. The time just before the calcium signal started to rise was set to time 0. The signal was integrated for 5 min from time 0.

Soluble TCR Production. The two chains were purified separately from inclusion bodies expressed in BL-21 CodonPlus cells (Stratagene) and solubilized in 8 M urea. The TCR was folded in vitro in 100 mM Tris (pH 8) buffer, 400 mM L-arginine, 0.5 mM oxidized glutathione, 5 mM reduced glutathione, and 2 mM EDTA. The folded protein was dialyzed four times in 10 mM Tris·HCl (pH 8), purified on a Hitrap DEAE column and MonoQ anion exchange columns (GE Lifesciences), and biotinylated with biotin-ligase (BirA) (Avidity). The protein was further purified by a HiLoad S200 gel filtration column (GE Lifesciences).

ERK Phosphorylation Assays. T2/A2–K^b or B16/A2–K^b cells were loaded with 10 μM gp209–2M peptide for 2 h at 37 °C. Splenocytes transduced with gp209-specific TCRs were labeled with CellTracker Blue (10 μM; Invitrogen) and fixable viability dye 660 (eBioscience) for 10 min at 37 °C in PBS. T cell/APC conjugate formation was initiated by quick centrifugation (10 s at $400 \times g$) of T cells and APC (1:1 ratio) followed by incubation at 37 °C for 5 min. The reaction was stopped on ice for 30 s. The plate was centrifuged at $311 \times g$ for 2 min, and the supernatant was discarded. T-cell/APC conjugates were disrupted by vortexing and fixed by 4% ice-cold paraformaldehyde (Electron Microscopy Sciences) for 15 min on ice. Cells were washed twice with FACS buffer (5% FBS, 1× PBS), permeabilized with 90% ice-cold methanol for 15 min on ice, and then washed twice again in FACS buffer. Cells were stained with primary antibody anti-mouse phosphorylated ERK (ppERK) (Cell Signaling; 1:1,000 dilution) for 30 min at room temperature and then washed twice in FACS buffer. Cells were stained with secondary antibody Alexa 546 anti-mouse IgG (Invitrogen) for 30 min at room temperature and washed twice in FACS buffer. Cells were analyzed on a LSR II Flow Cytometer equipped with a plate reader (BD Biosciences). The data were analyzed in Flowjo (Tree Star). Cells were first

gated based on forward and side scattering, then by CellTracker Blue staining (to select T cells), and then by viability dye staining (to eliminate dead cells).

Adoptive Cell Therapy. A2–K^b transgenic mice (female, 4- to 7-wk old) were purchased from Taconic and housed in an NYU animal facility. Then, 2×10^5 B16/A2–K^b tumor cells in 0.2 mL of PBS were injected in the flank region. One week later, the injected mice were irradiated with 500 cGy, randomized into seven groups of six mice each, and administered 10–20 $\times$ 10^6 transduced cells in 0.2 mL of PBS through tail vein injection. The number of cells injected was adjusted based on transduction efficiency of TCR so that each mouse received a similar number of cells expressing TCRs. All mice except for the control group also received 2×10^7 PFU of gp209–2M recombinant fowlpox virus (2) through the tail vein and five doses of 200,000 IU of recombinant human IL-2 (Novartis) intraperitoneally for 3 d. The tumor was measured by a caliper twice per week. The mice were killed when the tumor area reached >300 mm^2. Mice were housed in specific pathogen-free conditions at the Smilow Research Center Animal Facility (NYU). All animal experiments were performed in accordance with protocols approved by the NYU Institutional Animal Care and Use Committee.

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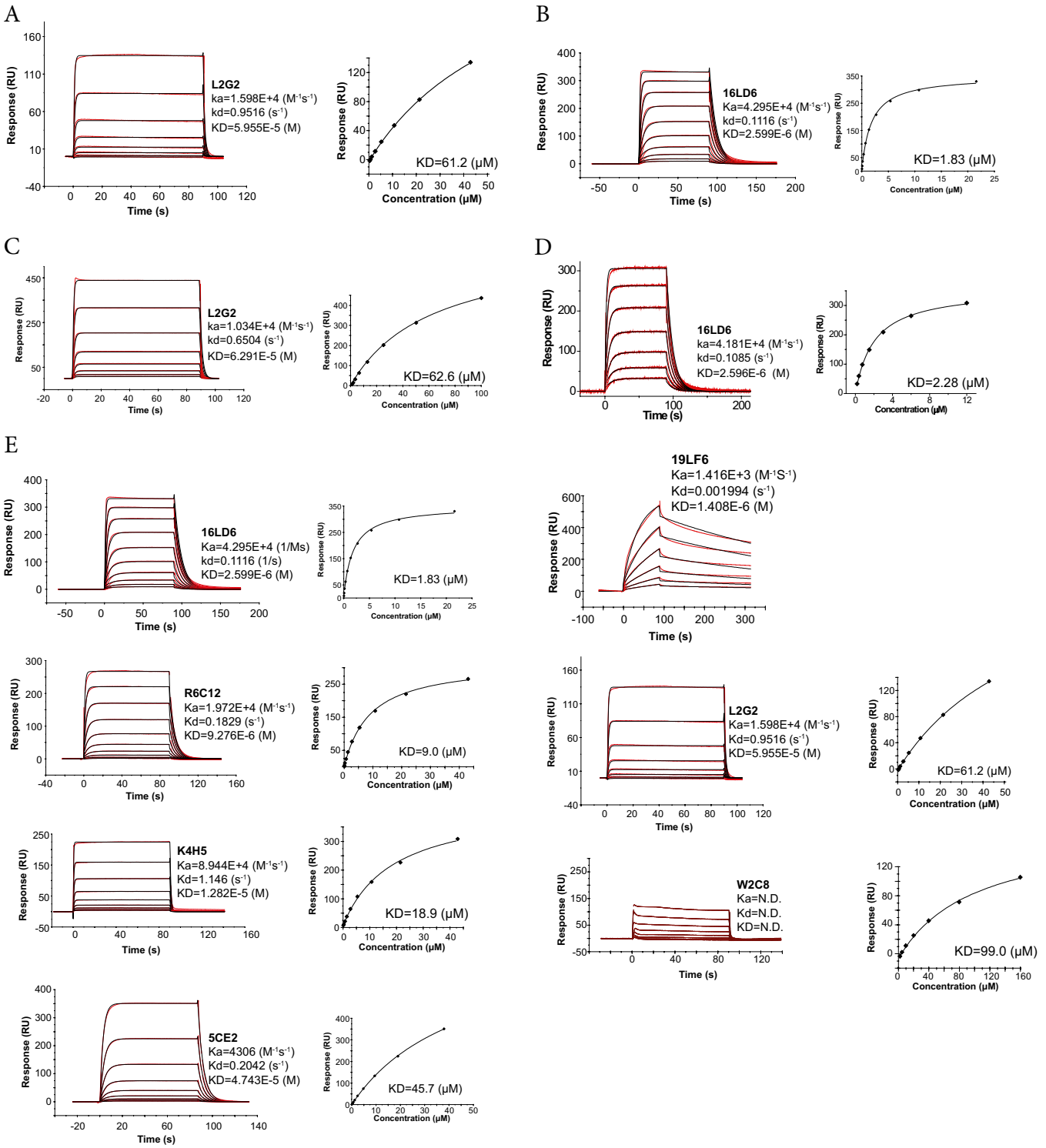

Fig. S1. Surface plasmon resonance (SPR) measurements of soluble gp209-specific TCRs. The TCRs were expressed as soluble proteins. An interchain disulfide bond was engineered between TCR α- and β-chains to facilitate pairing (1). A BirA tag was engineered on the C-terminal of the α-chain for site-specific biotinylation and immobilization. The accuracy of SPR measurements was limited by the accuracy of analyte concentrations. By immobilizing TCR molecules, the kinetic measurements are independent of TCR concentrations and thus give more accurate comparison between different TCRs. Immobilization through TCR α-chain also avoids TCR β homodimers, which are difficult to separate from the αβ heterodimers. (*A*–*D*) To verify that this strategy did not alter the binding kinetics, we compared the binding kinetics obtained with TCR immobilization (*A* and *B*) to commonly used peptide–major histocompatibility complex (pMHC) immobilization (2, 3) (*C* and *D*). The results are comparable for the two TCRs, L2G2 (*A* and *C*) and 16LD6 (*B* and *D*), tested. The binding kinetics were measured on a Biacore T100 machine. The kinetic data (red lines) were fitted with a 1:1 Langmuir binding model (black lines). Equilibrium constants were also obtained by fitting the steady states. (*E*) SPR measurements of soluble gp209-specific TCRs. The data were obtained by using TCR immobilization as described above. The kinetic data of all seven TCRs are shown, and the kinetic parameters obtained from the fitting are listed in Table 1.

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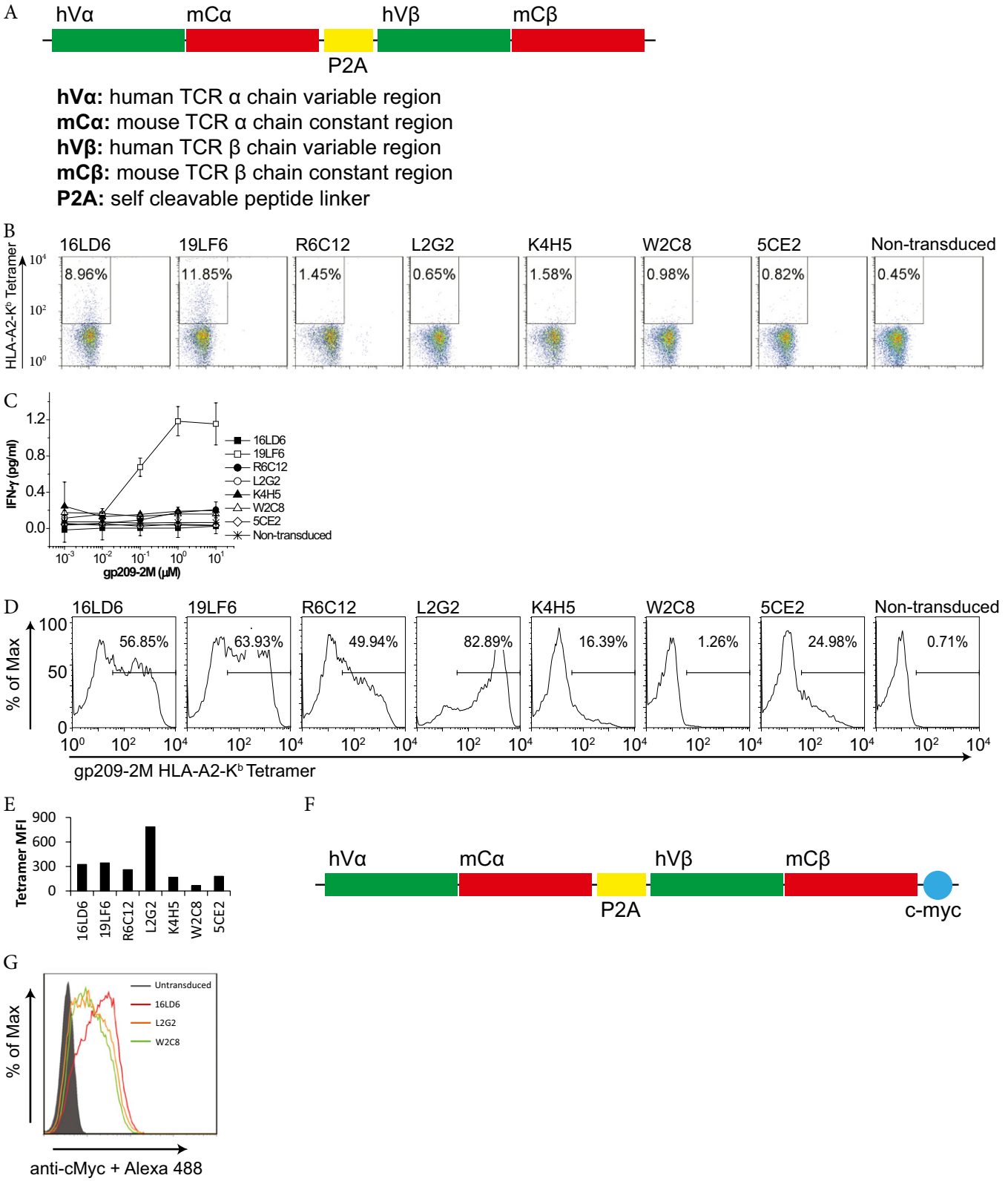

Fig. S2. Characterization of gp209-specific TCRs. (*A*) Full-length human TCRs failed to express on mouse T cells probably because human constant domains do not integrate with murine CD3, which is required for TCR surface expression. Therefore, we created hybrid TCR constructs (1): The human constant domains

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were replaced with the murine ones, and a self-cleavable P2A peptide linker (2) connects TCR α- and β-chains for stoichiometric expression. (*B*) gp209–2M–HLA-A2–K^b tetramer binding of CD8− splenocytes transduced with gp209-specific TCRs. The cells were generated by depleting CD8+ T-cell population from total splenocytes using anti-CD8β antibody staining and flow cytometry sorting (*SI Methods*). The majority of the cells (>90%) were CD4 T cells. (*C*) IFN-γ production of CD8− splenocytes transduced with gp209-specific TCRs mixed with T2/A2–K^b cells loaded with gp209–2M peptide. The cells were generated as in *B*. Error bars represent the SD of triplicate measurements. (*D*) gp209–2M–HLA-A2–K^b tetramer stainings of CD8+ splenocytes transduced with gp209-specific TCRs. CD8+ T cells were generated as described in *SI Methods*. (*E*) Mean fluorescent intensities (MFIs) of tetramer stainings from *D*. (*F*) Because no TCR-specific antibody is available for L2G2, the expression of L2G2 cannot be quantified through anti-TCR staining on splenocytes. To confirm that the expression level of L2G2 is similar to other TCRs on splenocytes, alternative constructs with a c-Myc epitope tag at the β-chain were created. (*G*) Intracellular staining of the c-Myc tag (*SI Methods*) demonstrated that L2G2 expression level was comparable to 16LD6 and W2C8, two selected TCRs with known expression levels (Fig. 1*A*).

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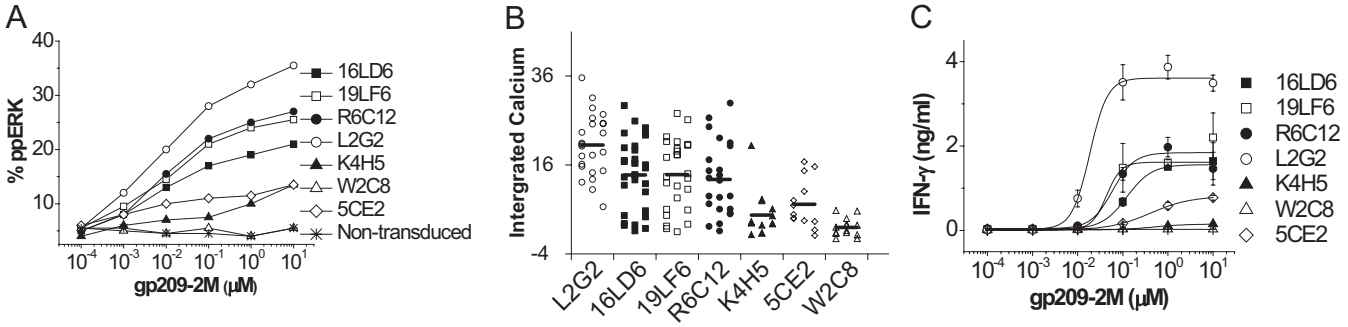

Fig. S3. The strength of the proximal and distal T-cell response. (*A*) The percentage of ERK phosphorylation of CD8+ splenocytes mixed with T2/A2–K^b cells loaded with gp209-2M peptide was measured by flow cytometry (Fig. S4 *A* and *B*). (*B*) Calcium release assays of transduced CD8+ splenocytes. T cells were labeled with calcium indicator dye Fura-2 and mixed with T2/A2–K^b cells loaded with 10 μM gp209–2M peptide. Elevated calcium signal of T cells forming conjugate with T2/A2-K^b cells was integrated for 5 min. Each symbol represents the integrated calcium signal of an individual T-cell/APC conjugate. The lines represent the average of the integrated calcium signal. (*C*) IFN-γ release assays of transduced CD8+ splenocytes. T cells were mixed with T2/A2–K^b cells loaded with gp209–2M peptide overnight at 37 °C, and the amount of secreted IFN-γ was measured by ELISA. The lines represent fittings with a logistic function in OriginPro (OriginLab). Error bars represent the SD of triplicate measurements. Data are representative of two independent experiments (*A* and *C*) or were pooled from two to eight independent experiments (*B*).

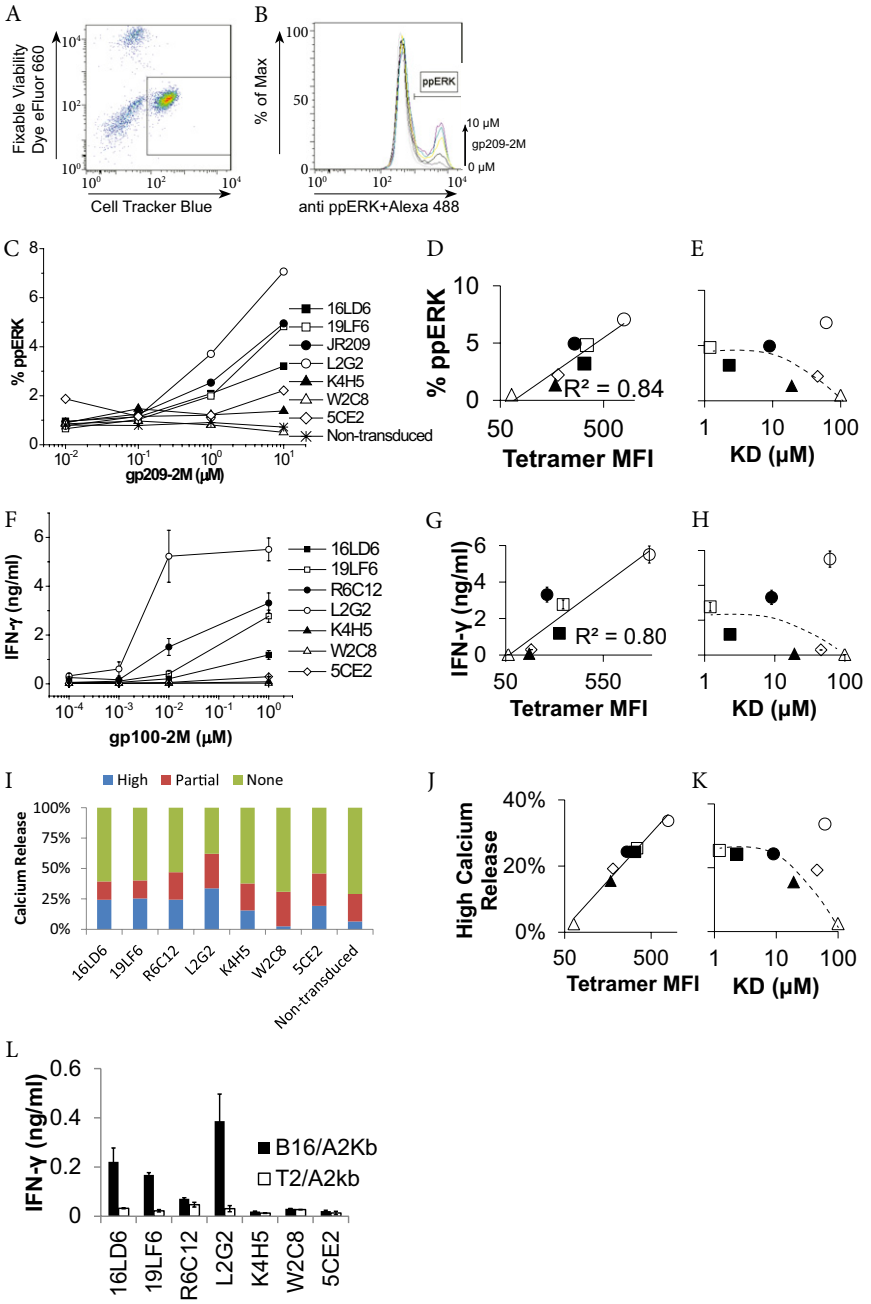

Fig. S4. The strength of proximal and distal T-cell response is correlated with TCR avidity and affinity. (*A*) Example of ERK phosphorylation assay by flow cytometry. T cells were stained with CellTracker Blue, anti-ppERK antibody, and fixable viability dye and gated on CellTracker Blue positive and fixable viability dye-negative population to obtain viable T-cell population. (*B*) The cells were further gated on anti-ppERK antibody-positive population to obtain percentage of T cells with phosphorylated ERK. (*C*) Percentage of ERK phosphorylation of CD8+ splenocytes mixed with B16/A2–K^b cells loaded with gp209–2M peptide for 5 min at 37 °C. Error bars represent the SD of triplicate measurements. (*D* and *E*) The percentage of ERK phosphorylation at a representative gp209-2M concentration (10 μM) was correlated with TCR avidity (*D*) or affinity (*E*). (*F*) IFN-γ release assays of transduced CD8+ T cells. T cells were mixed with B16/A2–K^b cells loaded with gp209–2M peptide overnight at 37 °C, and the amount of secreted IFN-γ was measured by ELISA. Error bars represent the SD of triplicate measurements. (*G* and *H*) The amounts of IFN-γ secretion at a representative gp209–2M concentration (1 μM) were correlated with TCR avidity (*G*) or affinity (*H*). (*I*) Percentage of T cells in contact with B16/A2–K^b cells loaded with 10 μM gp209–2M peptide showed elevated calcium signal. (*J* and *K*) T-cell calcium release was categorized as high, partial (transient calcium signal), and none according to a described method (1). The percentage of T cells with a high calcium signal correlates with TCR avidity (*J*) or affinity (*K*). (*L*) Cytokine productions of transduced CD8+ T cells mixed with unpulsed B16/A2–K^b or T2/A2–K^b cells.

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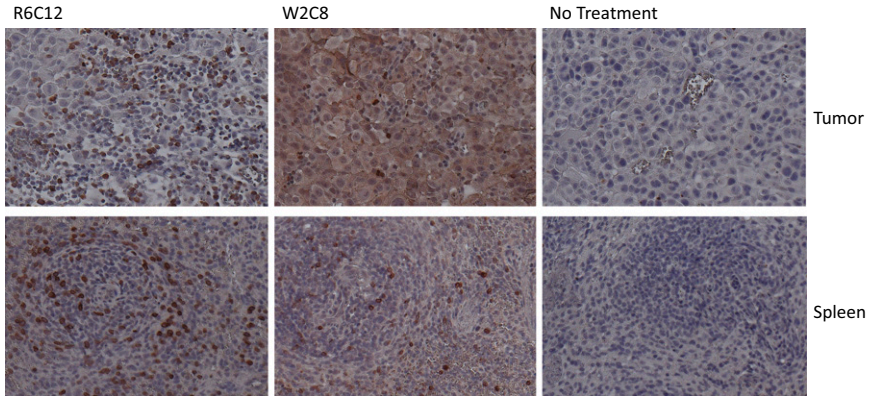

Fig. S5. Tumor and spleen from mice receiving adoptive cell therapy (ACT) for 5 d (Fig. 4) were stained with anti-2A antibody specific for the 2A epitope tag.

Table S1. Comparison of T-cell receptors in ACT treatment of melanoma patients

TCR	K_D, μM	Objective cancer regressions, % (*n*)	Cellular infiltrate into the eye, %
DMF4	29 (ref. 1)	13 (17) (ref. 2)	0 (ref. 2)
DMF5	5.6 (ref. 1)	30 (30) (ref. 3)	55 (ref. 3)

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