IL-2 is inactivated by the acidic pH environment of tumors enabling engineering of a pH-selective mutein

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Published in:
Science Immunology

DOI:
10.1126/sciimmunol.ade5686

Publication date:
2022
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Other Supplementary Materials for this manuscript include the following:

Movies S1 to S5
Data S1 - Normalized counts of DE top variable genes
Data S2 – DE cluster marker
Data S3 – DE Switch-2 vs IL-2 by cluster
Microscale thermophoresis (MST)

MST was conducted using a NT.115 Pico MST instrument (Nano Temper Technologies GmbH) equipped with red and blue filter sets. IL2 and Switch-2 were diluted to 200 nM in PBS buffer with 0.05% Tween (PBS-T) and labelled with Monolith His-Tag Labeling Kit RED-tris-NTA (Nano Temper; MO-L018). The RED-tris-NTA dye was diluted in PBS-T to 100 nM. The mix was incubated at room temperature (RT) in the dark for 30 min. 14 µM IL-2Rα or IL-2Rβ ectodomain were diluted with a serial 1:1 ratio of 16 gradients. Then the labelled protein and IL-2Rα ectodomain were mix with 1:1 ratio and incubated at RT in the dark for 15 min. Capillaries are then filled individually and loaded into instrument. Data were acquired using medium MST power and 20% LED. Data were analyzed using MO Control Software (Nano Temper). MST figures were rendered using MO Affinity Analysis (Nano Temper) and Prism 9 software (GraphPad).

Single-molecule fluorescence imaging

For microscopy experiments, HeLa cells stably transfected with SNAPf-IL-2Rα were transferred onto 25 mm glass coverslips coated with a poly-L-lysine-graft-(polyethylene glycol) copolymer functionalized with RGD to minimize non-specific binding (55). Single-molecule imaging experiments were conducted by total internal reflection fluorescence (TIRF) microscopy with an inverted microscope (Olympus IX71) equipped with a triple-line total internal reflection (TIR) illumination condenser (Olympus) and a back-7 illuminated electron multiplied (EM) CCD camera (iXon DU897D, Andor Technology) as previously described in more detail (41, 56). A 150 × magnification objective with a numerical aperture of 1.45 (UAPO 150 × /1.45 TIRFM, Olympus) was used for TIR illumination of the sample. All experiments were carried out at RT in medium
without phenol red, supplemented with an oxygen scavenger and a redox-active photoprotectant to minimize photobleaching (57).

For selective cell surface labelling of SNAPf-tagged IL-2Rα, cells were incubated at 37 °C with 80 nM of a premixed solution of 99.5 mol% SNAP-Surface 488 and 0.5 mol% SNAP-Surface 547 for 15 min and washed 5 times with pre-warmed PBS to remove unreacted dyes. Thus, a small and well-defined fraction of IL-2Rα was labelled with SNAP-Surface 547, allowing reliable quantification by single molecule localization microscopy. Dy647-labeled IL-2 and Switch-2 were added at a concentration of 1 nM 5 min prior to imaging experiments.

For single molecule experiments, orange (SNAP-Surface 547) and red (DY647) emitting fluorophores were simultaneously excited by illumination with a 561 nm fiber laser (2RU-VFL-P-500-560-B1R, MPB Communications) and a 642 nm fiber laser (2RU-VFL-P-500-642-B1R, MPB Communications). Fluorescence was filtered by a penta-band polychroic mirror (zt405/488/561/640/730rpc, Semrock) and excitation light was blocked by a penta-band bandpass emission filter (BrightLine HC 440/521/607/694/809, Semrock). For simultaneous acquisition of both channels with a single back-illuminated EMCCD camera (iXon Ultra 897, Andor Technologies), a four-color image splitter (QuadView, QV2, Photometrics) was used, which was equipped with three dichroic beamsplitters at 565 nm, 630 nm, and 735 nm (480dcxr, 565dcxr, 640dcxr, Chroma) and four single-band bandpass emission filters (BrightLine HC 438/24, BrightLine HC 520/35, BrightLine HC 600/37, BrightLine HC 685/40, Chroma). Image stacks of 150 frames were recorded for each cell at a time resolution of 32 ms/frame.

Single-molecule localization was carried out using the multiple-target tracing (MTT) algorithm (58). For comparing IL-2 binding, the number of molecules localized in the DY647 channel (IL-2
or Switch-2) during 30 consecutive frames were normalized with respect to the number of molecules localized in the SNAP-Surface 547 channel (IL-2Rα).

**Nano differential scanning fluorimetry (NanoDSF)**

IL2 and Switch-2 (10 µM) were analyzed using Tycho NT.6 (NanoTemper, Munich, Germany) by applying a standard capillary (10 µl) for each HBS buffer condition (pH 7 to 4). Thermal unfolding profiles were recorded within a temperature gradient between 35 °C and 95 °C. Inflection temperatures (Ti) values were determined automatically using the integrated software.

**Molecular modelling**

We ran atomistic molecular dynamics simulations of four different systems: the IL-2-IL-2Rα complex at pH 6 and 7 and the Switch-2-IL-2Rα complex at pH 6 and 7. We used the crystal structure of the full cytokine receptor complex (PDB: 2B5I) (39) to build the initial structure of the IL-2-IL-2Rα complex. Missing loops were built in using Modeller (59) to match the canonical sequence on Uniprot, but missing terminal regions were not added. In the starting structure of Switch-2, mutations were introduced using the mutagenesis tool in PyMOL. The protonation states of all histidines except His120 in IL-2Rα were set corresponding to pH 6 or pH 7 based on PROPKA (60) predictions; any histidine with a predicted pKa greater than the given pH was protonated. Independently of PROPKA prediction, His120 in IL-2Rα was protonated at pH 6 and deprotonated at pH 7 to test the effect on the dissociation rate of the complex. In neutral histidines, the hydrogen was placed on the ε nitrogen. Because we already observed increased stability of the complex at pH 7 with hydrogens placed on the ε nitrogens in neutral histidines, we did not run the simulations with hydrogens on the δ nitrogens in neutral histidines.

We used the DES-amber force field (61) and TIP4P-D water model (62) with Gromacs 2021.1 (63). Na⁺ or Cl⁻ ions were added only to neutralize the system. We use a cut-off of 1.0 nm for Van
der Waals interactions, and electrostatic interactions were treated using the Particle mesh Ewald method with a Fourier spacing of 0.16 nm, cubic interpolation, and a real-space cut-off of 1.0 nm. Simulations were run in the NPT ensemble at a temperature of 340K and a pressure of 1 bar using the Velocity-Rescaling thermostat (64) and Parinello-Rahman barostat (65). The high temperature of 340K was used to favor a more rapid dissociation of the complex. Energy minimization was performed for 50,000 steps using the steepest descent algorithm. Five replicas were started for each of the four systems. Each replica was equilibrated for 3 ns with a 0.2 fs timestep followed by 4 µs of production simulation with a 2 fs timestep.

To follow dissociation of the complex, we determined the minimum distance between any pair of atoms in the interaction interface using the Gromacs mindist function. The interaction sites were defined based on the crystal structure as Thr37-Phe44 and Glu62-Ala73 in IL-2 and Gly23-Glu29, Arg35-Leu45, and Arg117-Phe121 in IL-2Ra.

For testing the protein unfolding, we calculated the backbone RMSD over time for IL-2 and the IL-2 binding domain of IL-2Ra using MDTraj (66). For IL-2 the RMSD was calculated over all residues. For IL-2Ra, we defined the IL-2 binding domain as residue Thr24-Phe121, but excluded the large loop from residue Ala65-Gly102.

**Cell lysis and Western blot**

Stimulated cells were lysed with RIPA buffer with protease inhibitors (Cell Signaling Technology, 9806S) and phosphatase inhibitors (Santa Cruz biotechnologies, sc-45045 and sc-45065) for 15 min on ice. Lysates were quantified and 25 µg were loaded on acrylamide gels. Proteins were separated by SDS-PAGE electrophoresis and transferred on PVDF membrane (Merk Millipore, IPVH00010). Staining was performed with the following antibodies diluted in TBS/0.05% Tween-20 with 5% BSA: 1:1,000 anti-human phospho-STAT5 (clone D47E7; Cell Signaling Technology,
4322), 1:1,000 anti-human STAT5 (clone D206Y; Cell Signaling Technology, 94205), 1:2,000 anti-human ph-phospho-S6R (clone D57.2.2E; Cell Signaling Technology, 4858), 1:1,000 anti-human S6R (clone 5G10; Cell Signaling Technology, 2217). Anti-rabbit IgG-HRP (Cell Signaling Technology, 7074) was used at 1:6,000 in TBS/0.05% Tween-20 with 5% BSA. Chemiluminescent signal was detected using ECL prime western blotting detection reagent (GE Healthcare, 28990926) and images were acquired using LAS4000 (GE Healthcare).

**IL-2Rα CRISPR knock-out (KO)**

For CRISPR KO, Alt-R S.p. Cas9 Nuclease v3 (IDT, 1081058), Alt-R CRISPR-Cas9 tracrRNA (IDT, 1072533), and GAAACTCTAGCCACTCGTCC guide RNA (IDT) were used to generate the RNP complex following manufacturer’s instructions. 10^6 pre-activated CD8+ T cells resuspended in 20 µl of P3 buffer (P3 primary cell 4D-Nucleofector X kit; Lonza, V4XP-3032) were nucleoporated using program EO-115 of Amaxa 4D (Lonza) with RNP complex. IL-2Rα KO CD8+ T cells were expanded using 20 ng/ml IL-15 (Miltenyi, 130-093-955) for 3 days and rested O/N before IL-2 stimulation.

**Luminex analysis**

Cell supernatants were measured on a custom 36-multiplex R&D systems Luminex panel (R&D Systems) in the Immunoassay Biomarker Core Laboratory, University of Dundee. The samples were diluted two-fold following the assay instructions. A Bio-Plex Pro wash station was used to perform the wash steps. The multiplex assay plate was measured on a Bio-plex 200 analyzer using Bio-plex Manager software v6.1. 36 different analyte-specific antibodies are pre-coated onto microparticles.

Standards, samples, and a cocktail of all the microparticles were added to each well. The plate was covered with a foil plate sealer and left to incubate, shaking at 800 ± 50 rpm, for 2 h at RT. During
this stage immobilized antibodies bind the analytes of interest. Using the Bio-Plex Pro wash station, the plate was washed three times as according to assay instructions. Diluted biotinylated antibody cocktail specific to the analytes of interest was added to each well. The plate was covered with a foil plate sealer and left to incubate, shaking at 800 ± 50 rpm, for 1 h at RT. After this the plate was washed as before. Diluted streptavidin-phycoerythrin conjugate (Streptavidin-PE) was then added to each well. The plate was covered with a foil plate sealer and left to incubate, shaking at 800 ± 50 rpm, for 30 min at RT. After this the plate was washed as before. The microparticles were resuspended in wash buffer. The plate was placed on a plate shaker for 2 min set at 800 ± 50 rpm. The plate was read immediately using a Bio-plex 200 analyzer. The mean blank Median Fluorescence Intensity (MFI) was subtracted from the mean duplicate MFI readings for each of the standards and samples. A five-parameter logistic (5-PL) curve-fit standard curve was generated for each analyte using the Bio-plex Manager v6.1 software. The software also calculated the results considering the sample dilution.

**Murine CD8+ T cell isolation and activation**

Murine CD8+ T cells were isolated from mouse spleen using MagniSort mouse CD8 T cell enrichment kit (eBioscience; 8804-6822-74). Cells were activated for 3 days in complete media using coated anti-mouse CD3 antibody (Clone 145-2C11; Biolegend; 100340) and 2 µg/ml soluble anti-mouse CD28 antibody (Clone 37.51; Biolegend; 102116).

**Histology**

Lungs used for hematoxylin/eosin staining were fixed O/N in PBS/4% PFA and dehydrated in 30%, 50%, and then 70% ethanol. Hematoxylin/eosin staining was performed following standard protocol.

**References and Notes**


Supplementary figure 1

A. Cell stimulation → Sample barcoding → Pool samples and stain → Flow cytometry

B. IL-2 signaling kinetics

C. IL-15 dose-response

D. pH-sensitive cytokines

E. CD8+ T cells

F. YT cells

G. K_d values

H. K_d values
Fig. S1. IL-2 sensibility to low pH is IL-2Rα dependent.

(A) Workflow of T cell stimulation, fluorescence barcoding, and gating strategy applied to analyze the samples by flow cytometry. (B) Signaling kinetics of STAT5 phosphorylation in pre-activated CD8+ T cells stimulated with IL-2 in pH 7.5 or 6.5 at suboptimal (10 pM) and saturating dose (10 nM) (bottom panel). (C) Dose-response of IL-15-mediated STAT5 phosphorylation in pre-activated CD8+ T cells at pH 7.5 and 6.5. (D) Effects of pH on canonical cytokine-induced signaling. Pre-activated T cells or freshly-isolated monocytes were stimulated with the indicated cytokines at pH 7.5 and 6.5, and effect of acidic pH on levels of phosphorylated STAT3 (IL-6, IL-10, IL-21, IL-27, G-CSF), STAT5 (IL-15, GM-CSF) and ERK (IL-18) were measured after 15 minutes by flow cytometry. Percentage change in MFI relative to median measurement at pH7.5 shown. Pre-activated T cells were used to assay signaling by IL-6, IL-10, IL-21, IL-27, and IL-15, and monocytes were used to assay signaling by G-CSF and GM-CSF. (E, F) Expression of IL-2Rα (left panel) and dose-response of STAT5 phosphorylation (right panel) in non-pre-activated and pre-activated CD8+ T cells (E), and IL-2Rα+ or IL-2Rα- YT cells (F) stimulated with IL-2 in pH 7.5 or 6.5. (G, H) Microscale thermophoresis (MST) analysis of IL-2 interaction with IL-2Rα (G) IL-2Rβ (H) at different pH. For each condition, the K_d values are indicated. Dara are mean of two (C) or three (B, D-F) independent experiments with two technical replicates each.
**Supplementary figure 2**

**A** B16.SIY WT (s.c.) \( \text{PBS or 20 } \mu\text{g Fc-IL-2 (i.p.)} \)

Day 3 0 5 10 200mmol/l NaHCO\(_3\) (continuous treatment)

**B**

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**C**

![Survival Graph](image)

**D** B16.SIY WT or LDH KO (s.c.) \( \text{Phenotyping} \)

Day 0 5 10 15 20 30 PBS or 20 \( \mu\text{g Fc-IL-2 (i.p.)} \)

**E**

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<tr>
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**F**

![Flow Cytometry Graph](image)

**G**

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<tr>
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**Fig. S2.** Acidic pH reduces the efficacy of IL-2 therapy.

(A) Schematic representation of experiments. Mice were treated with 200 mmol/l NaHCO\(_3\) in the drinking water starting from 3 days prior to i.p. tumor inoculation until the end of the experiment.
When the tumor reached a size of 50-100 mm³ mice were randomized and treated with i.p. injections of 100 μl PBS or 20 μg of Fc-IL-2 daily for the subsequent 5 days. (B, C) Individual follow-up of mean tumor growth (B) and Kaplan-Meier survival curves (C) of mice engrafted with B16.SIY WT tumors following treatment (n=12). (D) Schematic representation of experiments performed as in D. Established B16.SIY WT or LDH DKO tumor (50-100 mm³) bearing mice were treated with daily i.p. injections of 100 μl PBS or 20 μg of Fc-IL-2 for 5 days. (E) Individual follow-up of mean tumor growth of mice engrafted with B16.SIY or B16.SIY LDH DKO tumors. (F) Representative gating strategy for the analysis of tumor infiltrating CD8+ and CD4+ T cells. (G-I) Percentage of CD8+ T cells (G), Treg cells (H), and ratio between CD8+ T and Treg cells (I) measured by flow cytometry. Data are the pooled results of two (B, C, E) or three (G-I) experiments. Statistical significance was determined by Log-rank test with Bonferroni’s correction (C), or by one-way ANOVA with Tukey’s correction (G-I). Each symbol represents a single tumor (G-I).
Fig. S3. Characterization of Switch-2 variant. (A) Table summarizing the amino acid residues targeted in the library and the residues found in the Switch-2 mutant. Residues specific of the Switch-2 are highlighted in red. (B) Structure of IL-2-IL-2Rα receptor complex. IL-2Rα and IL-2 are shown in yellow and blue, respectively. The mutations identified within Switch-2 are
highlighted in red and indicated in the right panel. (C, D) Microscale thermophoresis (MST) analysis of Switch-2’s interaction with IL-2Rα (C) and IL-2Rβ (D) at different pH conditions. For each condition, the $K_d$ values are indicated. (E) IL-2 and Switch-2 stability at different pH. The inflection temperatures are indicated.
Supplementary figure 4

A

B

C

Replica 4:

Replica 5:
**Fig. S4. Structure of Switch-2 variant.**

(A) A 2mFo–DFc map, contoured at 1 sigma, showing the region around the mutation sites in the Switch-2 structure. The electron density is represented in blue mesh. (B) Molecular dynamics simulations of the IL-2-IL-2Rα complex and Switch-2-IL-2Rα complex with histidine side chains protonated corresponding to pH 6 and 7. Five independent simulations, each 4 µs long, of each system were run. The structural stability of the complexes was monitored by calculating the minimum distance between any pair of atoms in the interaction interface. (C) Selected frames from molecular dynamics simulations of IL-2-IL-2Rα at pH 6. Frames from replicas 4 and 5 were selected as this show dissociation of the complex. IL-2 is shown in blue and IL-2Rα in red with R38 and H120 highlighted in green. Plots on the right show the minimum distance between any pair of atoms in R38 and H120 (red) and the minimum distance between any pair of atoms in the interaction interface of the complex. Stippled lines correspond to the selected frames.
Supplementary figure 5

Fig. S5. Protein unfolding analysis.

(A) Backbone RMSD of IL-2 or switch-2 over the five replica simulations of each system (blue) and the minimum distance between any binding site residues in IL-2 (or Switch-2) and IL-2Rα (orange). (B) Backbone RMSD of the IL-2 binding domain of IL-2Rα over the five replica
simulations of each system (blue) and the minimum distance between any binding site residues in IL-2 (or Switch-2) and IL-2Rα (orange).
Supplementary figure 6

A. IL-2Rα- YT cells

B. IL-2Rα+ YT cells

C. CD8+ T cells

D. IL-2Rα KO CD8+ T cells

E. ERK1/2 pT202-pY204

F. S6R pS235/236

G. Akt pS734

H. pH 7.5 vs. pH 6.5

I. STAT5 pY694

20
Fig. S6. Switch-2-mediated signaling in T cells.

(A, B) Dose-response of STAT5 phosphorylation (pSTAT5) in IL-2Rα- (A) and IL-2Rα+ (B) YT cells stimulated with IL-2 or Switch-2 at neutral pH 7.5 or pH 6.5. (C) IL-2Ra expression (left panel) and pSTAT5 levels (right panel) in pre-activated WT or IL-2Rα KO CD8+ T cells upon saturating (10 nM) and sub-saturating (10 pM) doses of IL-2 or Switch-2. (D) Dose-response of pSTAT5 in pre-activated CD8+ T cells stimulated with IL-2 or Switch-2 at neutral pH 7.5 or pH 6.5 acidified with 12 mM HCl or 15 mM Lactic Acid (LA). (E-G) Dose-response curve of phospho-ERK1/2 (pERK1/2) (E), phospho-S6 Ribosomal protein (pS6R) (F), and phospho-Akt (pAkt) (G) in pre-activated CD8 T cells upon stimulation with IL-2 or Switch-2 at different pH conditioned media. (H) Western blot analysis of total and phosphorylated STAT5 and S6R levels in pre-activated CD8+ T cells upon stimulation with IL-2 and Switch-2 at different pH conditioned media. (I) Dose-response curve of pSTAT5 in Treg cells upon stimulation with IL-2 and Switch-2 at different pH. Data are the results of two (C, E-G, I) or three (A, B, D) experiments with two technical replicates each.
Supplementary figure 7

Fig. S7. Switch-2 effect on CD8+ T cells cultured in acidic pH.

(A, B) CD8+ T cells were labelled with cell trace violet (CTV) and activated with anti-CD3/CD28 activation beads under neutral and acidic pH conditions. Proliferation measured by 3-day CTV-dye dilution assay using flowcytometry. Representative flow plots of day 3 (A) and proliferation of CTV-labelled pre-activated CD8+ T cells cultured for three additional days under neutral and acidic pH conditions in the presence of 10 nM IL-2 or Switch-2 (B). Histograms represent the dilution of the CTV as a result of cell proliferation. One representative experiment out of three is shown. (C) Scatter plots of the proliferation index after 3 days culture (n=3). (D, E) Percentage of TNF-α and IFN-γ expression in CD8+ T cells stimulated with IL-2 or Switch-2 under different pH conditioned media that are re-stimulated with PMA + Ionomycin as in Figure 3B and the level of intracellular cytokines were measured using flow cytometry (n=5). Each symbol represents a
single donor. Statistical significance was determined by one-way ANOVA with Tukey’s correction.
Supplementary figure 8

A. Switch-2 vs IL-2 in human CD8+ T cells

B. Number of DEGs x 10^3

C. Allograft rejection
   IL-2 STAT5 signaling
   IL-6 JAK STAT3 signaling
   Inflammatory response
   Interferon alpha response
   Interferon gamma response
   KRAS signaling
   MTORC1 signaling
   MYC targets V1
   MYC targets V2
   TGFB signaling
   TNFα signaling via NFκB

D. Stim. - IL-2 - Switch-2

E. Stim. - IL-2 - Switch-2

F. Stim. - IL-2 - Switch-2
Fig. S8. Switch-2 at pH 6.5 induces transcriptional program similar to IL-2 at pH 7.5.

(A) GSEA analysis of Switch-2 versus IL-2-stimulated pre-activated CD8+ T cells at pH 7.5 (top panel) and at pH 6.5 (bottom panel) as in Fig 3C. (B) Number of differentially expressed genes (DEGs) that are up- or down-regulated upon stimulation with IL-2 or Switch-2 at different pH. (C) Hallmarks significantly enriched in each cluster obtained from the heatmap with the most variable genes (see Fig. 3D). (D-F) Heatmap of the genes contained within cluster 2 (see Fig. 3D). The genes contained in this cluster were divided in 3 different heatmaps representing the three main subclusters within cluster 2. Gene expression is represented as z-score.
Supplementary figure 9

Fig. S9. pH-dependent activity of Switch-2 in vivo.

(A) Dose-response curve of STAT5 phosphorylation at pH 7.5 or 6.5 induced by IL-2 and Switch-2 in pre-activated murine CD8+ T cells. Data were obtained from two independent experiments.
with two technical replicates each. (B, C) Plot showing the percentage of AF647+ cells in CD25- and CD25+ CD8+ T cells in tumor (B) and lung (C) (n=8). (D) CD25-dependent uptake of AF647 labelled Fc-IL-2 and Fc-Switch-2 in CD8+ T cells from mouse tumor draining lymph nodes (tdLN) and blood. (E, F) Plot showing the percentage of AF647+ cells in CD25- and CD25+ CD8+ T cells in tdLN (E) and blood (F) (n=8). (G) Schematic representation of in vivo STAT5 phosphorylation analysis. (H-L) Representative histogram plot (left panel) and plot of STAT5 phosphorylation (right panel) in CD8+ T cells from blood (H), tumor (I), and tdLN (L). Each dot represents a mouse. Data are pooled from two (I-L) or three (B, C, E, F) independent experiments. Significance was calculated by two-way (B, C, E, and F) or one-way (I-L) ANOVA with Tukey’s correction.
Fig. S10. *In vivo* anti-tumor efficacy of Switch-2 versus IL-2 in tumor models

(A) 7 days after tumor injection, when MC38 tumor reached a size of 50-100 mm$^3$, mice were treated with daily i.p. injections of 100 µl PBS or 20 µg of Fc-IL-2 or Fc-Switch-2 for 5 days. (B) Individual follow-up of tumor volume, depicting the mice that completely rejected the tumors. (C) 7 days after tumor injection, when 4T1 tumor reached a size of 50-100 mm$^3$, mice were treated with i.p. injections of 100 µl PBS or 20 µg of Fc-IL-2 or Fc-Switch-2 for 7 days. Mice were sacrificed at day 21. Individual follow-up of tumor volume (D) and mean tumor volumes comparing different treatment groups (E) (n=12 for PBS and IL-2 groups and n=11 for Switch-2-treated group). (F) Picture of 4T1 tumors from one representative experiment. (G) 7 days after tumor injection, when B16.SIY WT tumor reached a size of 50-100 mm$^3$, mice were treated with i.p. injections of 100 µl PBS or 20 µg of Fc-IL-2 or Fc-Switch-2 daily for 5 days. (H) Individual follow-up of mean B16.SIY WT tumor. (I) Schematic representation of experiments. Mice were treated with 200 mmol/l NaHCO$_3$ in the drinking water starting from 3 days prior to i.p. tumor inoculation until the end of the experiment. When the tumor reached a size of 50-100 mm$^3$ mice were randomized and treated with i.p. injections of 100 µl PBS or 20 µg of Fc-IL-2 or Fc-Switch-2 daily for the subsequent 5 days. Individual follow-up of tumor volume (J) and mean tumor volumes comparing different treatment groups (K) (n=12). (E, K) Results are pooled from two independent experiments. Significance was calculated by or one-way ANOVA with Tukey’s correction.
Supplementary figure 11

Fig. S11. anti-PD1 and Switch-2 combination therapy results in improved tumor control in B16.SIY model compared to anti-PD1+IL-2 combination therapy.

(A) Schema of IL-2 therapy in combination with anti-PD-L1 in B16.SIY model. B16.SIY WT-bearing mice were treated with i.p. injections of 100 µl PBS or 20 µg of Fc-IL-2 or Fc-Switch-2 for 5 days. At day 7, 10, and 13, mice were injected with 100 mg of anti-PD-L1 or control IgG. Treatment was started at day 7 when the tumor reached a size of 50-100 mm³. (B) Individual follow-up of tumor volume and mean tumor volumes (C) comparing different treatment groups and survival curve (D) of B16 tumors (n=12) treated as depicted in (A). Results are pooled from two independent experiments. Significance was calculated by one-way ANOVA with Tukey’s correction (C) or by Log-rank test with Bonferroni’s correction (D).
Supplementary figure 12

Fig. S12. Effect of Switch-2 therapy in B16-F10-mCherry-OVA metastatic melanoma model

(A) Schema of IL-2 therapy in B16-F10-mCherry-OVA metastatic model. 7 days after i.v. injection of B16-F10-mCherry-OVA, mice were treated with i.p. injections of 100 µl PBS or 20 µg of Fc-IL-2 or Fc-Switch-2 for 5 days. (B) Number of CD45-mCherry+ tumor cells in the lung pair of each mouse. (C, D) Representative pseudocolor plot of OVA tetramer staining CD8+ TILs (C) and percentage of OVA tetramer+ CD8+ TILs. (F-H) percentages of CD8+ (F), Treg (G), and NK cells (H). Each dot represents a single mouse (n=22 for PBS, n=14 for Fc-IL-2, and n=13 for Fc-Switch-2 group). Results are pooled from two independent experiments. Significance was calculated by or one-way ANOVA with Tukey’s correction.
Fig. S13. Analysis of Switch-2 effect on TILs in B16.SIY WT tumor model.

(A-E) Percentage of CD8+ (A), Treg cells (B), ratio between CD8+ and Treg cells (C), percentage of conventional CD4+ T cells (D), and NK cells (E) and Ki67+ proliferating CD8+ T cells, IFN-γ+ CD8+ T (G) and TNF+ CD8+ T (H) in established B16.SIY WT tumors treated with the IL-2 variants or PBS, measured by flow-cytometry (n=18), (I) Representative dot plots of CD62L and CD44 expression in CD8+ TILs (J) Expression of CD44 in CD8+ TILs after therapy (n=12). (K-
M) Representative dot plots of PD-1 and TIM3 expression in CD8+ TILs and percentages of PD-1+TIM3- (L) and PD-1+TIM3+ (M) CD8+ TILs (n=18). Each symbol represents two mice pooled together (G-H) or a single mouse (A-F, J, K, M). Data are pooled from two (J) or three (A-H, L, M) independent experiments. Significance was calculated by one-way ANOVA with Tukey’s correction.
Fig. S14. A critical role of LNs in mediating anti-tumor response induced by Switch-2

(A-C) Percentage of CD8+ (A), Treg (B), and conventional CD4+ T cells (C) in tdLN of B16 tumor-bearing mice from the indicated treatment groups (n=12). (D) Representative dot plots of SIY pentamer staining in CD8+ T cells from tdLN. (E) Percentage of SIY antigen specific CD8+ T cells in tdLN (n=12). (F) Representative dot plots of CD62L and CD44 expression in CD8+ T cells from tdLN. (G) Percentage of CD62L+CD44-, CD62L-CD44+, and CD62L+CD44+ CD8+ T cells in and tdLN (n=12). (H) Schema of illustrating the time points of IL-2 therapy and FTY720 treatment in B16.SIY model when the tumor reached a size of 50-100 mm³. Blue arrow heads indicate initiation of FTY720 treatment and was administered through the entire the experiments. Red arrow heads indicate treatment with IL-2 variants. (I) Individual follow-up of mean tumor volume (C) and mean tumor comparing no treatment or FTY720 treatment to PBS or IL-2 treatment groups. Each curve represents the tumor growth in one mouse. (J, K) Growth (J) and survival curve (K) of B16 tumors treated as depicted in (H) (n=12). Data are mean of two independent experiments. Statistical significance was determined by two-way (G) or one-way (A-C, E, J) ANOVA with Tuckey’s correction or by Log-rank test with Bonferroni’s correction (K).
Fig. S15 scRNA-seq analysis of CD8+ TILs after Switch-2 treatment in B16.SIY WT tumor model.

(A) Dot plot of selected markers used to name the clusters. (B) UMAP from scRNA-seq colored by expression of the indicated gene markers. (C) UMAP from scRNA-seq colored by sample. (D) Histogram plot of the fraction of cells in G1, S, G2M cell cycle phases in each sample.
Table S1. Primers for IL-2 library generation

List of the primers used to generate the IL-2 library for yeast display. Create a page break and paste in the Table above the caption.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>5’-3’ Sequence</th>
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<tbody>
<tr>
<td>Lib Fw</td>
<td>AGCGGTGGGGGCGGTTCTCTCTCT</td>
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<tr>
<td>Lib Rv</td>
<td>TCGAGCAAGTCTTTCTTCCGGAG</td>
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<tr>
<td>IL-2 Amp Fw</td>
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<td>TCGAGCAAGTCTTTCTTCCGAGATAAGCTTTTTGTCGCCACCAG AAGCGGCGCCAGTCAGTGTGGAGATGATGCT</td>
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Table S2. Crystallographic statistics for the CD25:Switch 2 complex.

Values in parentheses are for the highest resolution shell.

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<th>Data Processing</th>
<th>CD25 : Switch-2</th>
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<td>Unit cell</td>
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<td>Mean I/sigma(I)</td>
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<tr>
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<tr>
<td>Reflections used for R-free</td>
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<tr>
<td>R-free</td>
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<tr>
<td>Number of TLS groups</td>
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**Movie S1.**
Trajectories from 100 consecutive frames of simultaneous dual color imaging of IL-2 binding to IL-2Rα-expressing HeLa at pH 7.

**Movie S2.**
Trajectories from 100 consecutive frames of simultaneous dual color imaging of IL-2 binding to IL-2Rα-expressing HeLa at pH 6.

**Movie S3.**
Trajectories from 100 consecutive frames of simultaneous dual color imaging of Switch-2 binding to IL-2Rα-expressing HeLa at pH 7.

**Movie S4.**
Trajectories from 100 consecutive frames of simultaneous dual color imaging of Switch-2 binding to IL-2Rα-expressing HeLa at pH 6.

**Movie S5.**
Simulation of IL-2-IL-2Rα interaction at pH 6. The video shows the dissociation in replica 5 between 1.3 and 1.4 μs. Arg38 in IL-2 and His120 in IL-2Rα are highlighted in red.

**Data S1. (separate file)**
Normalized count of the top variable and differentially expressed genes upon IL-2 or Switch-2 stimulation at pH 7.5 or 6.5.

**Data S2. (separate file)**
Differentially expressed genes between each cluster and all other clusters pooled together using two-sided Wilcoxon rank-sum test. Adjusted $p$ value were computed using Bonferroni
correction. Only genes expressed in at least 10 percent of cells in either group, with an average absolute log2 fold change above 0.2 and with an adjusted p-value < 0.05 were considered differentially expressed. pct.1: proportion of cells expressing a gene in a cluster; pct.2: proportion of cells expressing a gene in all other clusters.

Data S3. (separate file)

Differentially expressed genes between Switch-2 and IL-2 samples in each cluster pooled together using two-sided Wilcoxon rank-sum test. Adjusted p-value were computed using Bonferroni correction. Only genes expressed in at least 10 percent of cells in either group, with an average absolute log2 fold change above 0.2 and with an adjusted p-value < 0.05 were considered differentially expressed. pct.1: proportion of cells expressing a gene in a cluster; pct.2: proportion of cells expressing a gene in all other clusters.