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Supporting Information for
Cytokine conjugation to enhance T cell therapy

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Animal Handling

Experiments and handling of mice were conducted under federal, state, and local guidelines and with approval from Harvard University's Institutional Animal Care and Use Committee (IACUC) and in compliance with National Institutes of Health (NIH) guidelines. Five- to six-week-old female C57Bl/6 mice, TCR-transgenic Thy1.1+ Pmel mice, and Nod/SCID/IL2RG$^{-/-}$ (NSG) mice were purchased from the Jackson Laboratory.

Preparation of G400 NP

Ac_3ManAzAL, the monomer of G400 was first synthesized as previously described (1). Ac_3ManAzAL (1.0 mmol), AIBN (0.0005 mmol), and PEG DDMAT (0.0025 mmol) were dissolved in anhydrous dimethylformamide, freeze-thawed three times, and stirred at 65°C for 48 hours. G400 polymer was then obtained with precipitation in cold diethyl ether and washing with cold diethyl ether for 3 times, and then dried for future use. G400 polymer was then dissolved in dimethylformamide at 40 mg/ml, and added dropwise to ultrapure water on vigorous stirring. After 2 hours, the G400 NP solution was dialyzed against deionized water for 48 h, concentrated with Amicon 3k centrifugal filters, and stored at 4°C for future use.

T cell isolation, activation, culturing, and azido-labeling

Mouse spleens were collected and kept on ice until and throughout processing. To obtain splenocytes, spleens were compressed and passed through a 70 μm strainer, washed with PBS, and centrifuged at 300 g for 5 min at 4°C. The pellet was resuspended in 1 ml ACK buffer (Lonza, #10-548E) for 1 min; 9 ml of PBS was then added and splenocytes collected by centrifuging at 300 g for 5 min at 4 ºC. Mouse Pan T cells and CD8+ T cells were isolated with magnetic-bead-based Pan T cell (Miltenyi #130-095-130) and CD8+ T cell (Miltenyi #130-104-075) isolation kit. Human PMBCs were isolated from blood samples obtained from the Specimen Bank at Brigham and Women's Hospital, and human T cell were isolated from PBMCs with magnetic-bead-based Pan T cell (Miltenyi #130-096-535) CD8+ T cell isolation kit (Miltenyi #130-096-495) following manufacturer protocol. Mouse and human CD8+ T cells were activated with Dynabeads mouse/human T-activator respectively (ThermoFisher Scientific #11452D/#11131D) and cultured in T cell media (RPMI 1640 (Lonza #BE12-702F), 10% heat-inactivate fetal bovine serum (Gibco #10-082-147), 1% pen/strep, 55 μM *β*-mercaptoethanol, 10 mM HEPES, 1% 100x non-essential amino acid (Lonza #13-144E), 100 mM sodium pyruvate (Lonza #13-115E), supplemented by mouse recombinant IL-2 (BioLegend #575406) or human recombinant IL-2 (BioLegend #589106) respectively (30-200 IU/ml). T cells were azido-labelled by adding G400 NP solution directly to T cell cultures at various concentrations for 72 hours after activation.

Flow cytometry analysis of azido-labeled T cells

G400 NP treated T cells were washed 3 times with PBS. For flow cytometry, T cells were stained for live/dead cells, washed with PBS, fixed, and stained with DBCO-AF594 at a concentration of 1 mM at 4°C for 30 minutes. Stained T cells were then analyzed with flow.

Imaging and Azido-signaling analysis of azido-labeled T cells with confocal imaging

T cells were treated with G400 NP for 3 days, after which T cells were washed 3 times with PBS, and kept in G400 NP-free media for culture. At each timepoint, T cells were taken, washed with PBS 3 times, fixed and stained with DBCO-AF594 at a concentration of 1 mM at 4°C for 30 minutes, and loaded onto coverslips with ProLong Gold Antifade mountant with DAPI for confocal imaging. Z-stacks of T cells were analyzed with IMARIS imaging analysis software. Cells were identified and added as elements for analysis based on DAPI (nuclei) and AF594 (cytosol) staining. The level of azido-labeling in each cell was determined by the median intensity of AF594 on the cell surface.

DBCO-modification of cytokines and verification

DBCO- IL-12, IL-21, TNF-α, and IL-15/IL-15Rα were obtained via reacting carrier-free cytokines with DBCO-sulfo-NHS at 1:8 molar ratio in PBS for 2 days at 4°C. The reaction mixture was then washed and concentrated with Amicon 3k centrifugal filters, and stored at -80°C for future use.

Conjugating DBCO-modified cytokines onto T cells

T cells were treated with 200μM G400 NP for 3 days, and washed with PBS 3 times. DBCO-cytokines were added to T cells for 30 minutes at 4°C. T cells were then washed 3 times with PBS before characterization or use. Cytokine-containing media before and after the reaction were collected, and ELISA was performed according to manufacturer's protocol to calculate the amount conjugated onto T cells.

Flow cytometry for *in vitro* and *in vivo* cell analysis

Cells were stained with dead cell stain (ThermoFisher Scientific #L23105) according to manufacturer's protocol. Cells were then blocked with FcX Fc receptor blocking solution (BioLegend #101319, #422301) for 5 min and stained with surface protein antibodies for 20 min. Brilliant violet staining buffer (BD Horizon #563794) and flow cytometry staining buffer (Invitrogen #00-4222-26) were used during staining. Flow cytometry was then performed on BD Fortessa LSRII. Gating was performed based on fluorescence-minus-one controls. For blood samples from mice, 100 µl of ACK lysis buffer was added to every 50 µl of blood for 2 minutes to remove red blood cells. The cells were then washed and used for downstream staining. Anti-mouse and anti-human antibodies were obtained from BioLegend: Anti-mouse: CD103 (#121420), CD11b (#101263), CD11c (#117308), CD137 (#106110), CD19 (#115538, #115534), CD24 (#101826), CD25 (#101908), CD3 (#100218, #100220), CD301b (#146814), CD4 (#100559), CD40 (#124630), CD40L (#106512), CD44 (#103057), CD45 (#103132, #103114), CD62L (#104418), CD69 (#104545), CD8 (#100766), CD80 (#104743), F4/80 (#123147, #123128), I-Ab (#116406), LAG-3 (#125212), Ly-6C (#128012),MHC-II (#116406), NK-1.1 (#108748), PD-1 (#135206), Thy1.1 (#202526), Thy1.2 (#140329), Tim3 (#134008). Anti-human: CD3 (#344808), CD4 (#344648), CD8 (#344714)

Cytolysis Assay

1 million mouse B16-F10 cells were stained with Calcein AM (Invitrogen #C1430) for 30 min at 37 °C in dark, washed 4 times with PBS, and kept in dark on ice until use. T cells from Pmel-1 mouse spleen were isolated, treated with G400 NP for 3 days and conjugated with various DBCO-cytokines. T cells and B16-F10 melanoma cells were well mixed and seeded in 96-well U-bottom plates at different E:T ratios in 200 µl media (RPMI 1640 + 10% FBS + 1% P/S). The plate was centrifuged to collect cells to the bottom, and incubated at 37 °C for 6 hr. Supernatants were collected and their fluorescent signals measured with 485 nm excitation and 528 nm emission with a BioTek Synergy H1 plate reader. The fluorescent signals from the 528 nm channel were subtracted from the 485 nm channel, and cytolysis efficiency was calculated using (sample well – background well) / (full-cytolysis well – background well) * 100%

CAR-T Generation

Lentivirus construction and production: The 2nd generation CD19 CAR construct was composed of the scFv fragment from the FMC63 antibody (GenBank: ADM64594.1) fused to the human CD8α hinge and transmembrane region (Gene bank number NP_001759.3, aa 138-206) and linked to human 4-1BB (Gene bank number NP_001552.2, aa 214-255) and human CD3ζ (Gene bank number NP_000725, aa 52-163) intracellular signaling domains. To enable detection by flow cytometry, a cleavable truncated EGFR (tEGFR) was inserted to the N-terminus of the CD3ζ. Lentiviral supernatants were produced using the HEK 293T packaging line as previously described32. 100% confluent HEK 293T cells in a well of a 6-well plate was co-transfected with 0.2 ug CAR-vector plasmid, 0.9 ug pMD2.G, 1.9 ug psPAX2 using lipofectamine 2000 (Life Technologies). The cultures were grown for 55 hr, after which the supernatants were collected, filtered to remove debris, and frozen at -80 C before use.

CAR-T transduction: T cells were isolated from healthy donors using the human pan-T cell isolation kit (Miltenyi Biotec) to obtain CD3+ T cells. Isolated T cells were activated with Dynabead (ThermoFisher Scientific 111161D) at 1:1 ratio. After 48 h, activated T cells were transduced by adding 140 µL of pre-warmed lentiviral supernatant containing the CD19 CAR construct. After 36 h, the media containing T cells and any remaining material were transferred to a 6-well G-Rex plate (Wilson Wolf) containing pre-warmed T cell media (described above) and expanded for 3 days. Transduced T cells were magnetically separated from Dynabeads and cryopreserved in 10% DMSO + 90% FBS. The CAR-T transduction efficiency was consistently around 25%.

B16-F10 melanoma model

Tumor inoculation and Pmel T cell treatment: 100k B16-F10 melanoma cells were injected subcutaneously on the left flank of C57Bl/6 mice (female, ~6 weeks of age, Jackson Laboratory). After 5 days, tumor bearing mice were randomized and injected with either PBS, or 7 million Thy1.1+ Pmel T cells via tail vein injection.

Tumor monitoring: size of B16-F10 melanoma was monitored over time by measuring the height, width, and length of the tumor. The total size of tumor was calculated as height*width*length*0.5. Mice were euthanized if the total tumor size was over 2,000 mm^3, if any of the tumor dimensions were over 20 mm, or if significant discomfort or weight loss was observed.

Tracking of adoptively transferred T cells: Each week, animals were bled via the tail vein and ~50 µL blood was collected in K2-EDTA-coated collection tubes (BD). The samples were treated with ACK lysis buffer (Lonza, BioLegend), washed, and processed for flow cytometry as described above.

Xenograft lymphoma model

Tumor inoculation and CAR-T cell treatment: Female NSG mice, between 6-7 wks of age were inoculated with a high dose of 5×10^5 luciferized Raji cells (Raji-luc) intravenously on day 0. After 4 days, tumor-bearing mice were randomized into treatment groups and were treated with either mock (PBS) or 5×10^5 CAR+ T cells.

Tumor tracking: Raji-luc tumor burden was monitored over time using D-Luciferin (Gold Biotechnology). Animals were anesthetized and intraperitoneally injected with D-Luciferin at 150 mg/kg. Luminescence was measured 10 minutes post injection via IVIS (Perkin Elmer). Total flux (p/s) per mouse was quantified in whole-body regions-of-interest (ROI). Animals were imaged once every 4 to 14 days. Mice were euthanized if flux from tumor was larger than 1E1, or if significant discomfort or weight loss was observed.

Tracking of adoptively transferred CAR-T cells. Each week, animals were bled via the tail vein and ~50 µL blood was collected in K2-EDTA-coated collection tubes (BD). The samples were treated with ACK lysis buffer (Lonza, BioLegend), washed, and processed for flow cytometry as described above.

Cell isolation from tumor and lymph node

Tumors and tumor-draining lymph nodes were separated from mice and mashed into small pieces. Mashed tissues were treated with 200 IU collagenase type I at 37°C for 1 hour, and were passed through pipette tips every 20 minutes. After collagenase type IV treatment, the solutions were passed through a 70 µM filter to achieve a single cell suspension. Cells from lymph node were then used for downstream analysis. Cells from tumor were spun down and treated with ACK lysis buffer to remove red blood cells, and resuspended in 1 mL RPMI media. This single cell suspension was gently added to the top of 2 mL 40% (v/v) Percoll and 2 mL 70% (v/v) Percoll to create a separation, and centrifuged at 800g for 30 minutes at room temperature. Tumor infiltrating lymphocytes were collected from the middle layer and washed with ice-cold PBS for three times before they were used for downstream analysis.

T cell activation assay and intracellular cytokine staining

Mouse T cells isolated from tumor, spleen, and lymph nodes were co-cultured with B16-F10 melanoma T cells in 100 µl media. The plate was centrifuged at 1600 rpm to collect cells to the bottom. After 1 hour, GolgiPlug (BD #555029) was added according to manufacturer's protocol to stop cytokine secretion. 3 hours later, T cell and tumor cell mixtures were washed, and stained with dead cell stain, surface proteins, and intracellular cytokines with Cyto-Fast fix-perm buffer set (BioLegend #426803) according to manufacturer's protocol. Antibodies against intracellular cytokines were obtained from BioLegend: TNF-α (#506306), IL-2 (#503837) IFN-γ (#505830), Granzyme B (#396404)

Tetramer staining

Blood samples from mice were treated with ACK lysis buffer to remove red blood cells. 10 µl of SIINFEKL tetramer (TB-5001-4) were added to each sample, the final volume was adjusted to 200 µl with PBS, and the sample was incubated for 20 min at 37°C. Primary antibodies for other cell surface markers were then added and incubated for 20 min at 4°C. Cells were then washed with PBS for three times and stained with live/dead staining, and used for flow cytometry analysis.

Statistical Analysis

Data was represented as mean ± SEM of biological replicates, unless otherwise stated. Detailed statistical methods are described in figure legends. Statistical testing was performed using GraphPad Prism (Version 9.02). $p < 0.05$ was considered significant.

Data, Materials, and Software Availability

All of the study data are included in the article and/or supporting information.

References

1. H. Wang, et al., Metabolic labeling and targeted modulation of dendritic cells. Nature Materials 2020 19:11 19, 1244–1252 (2020).

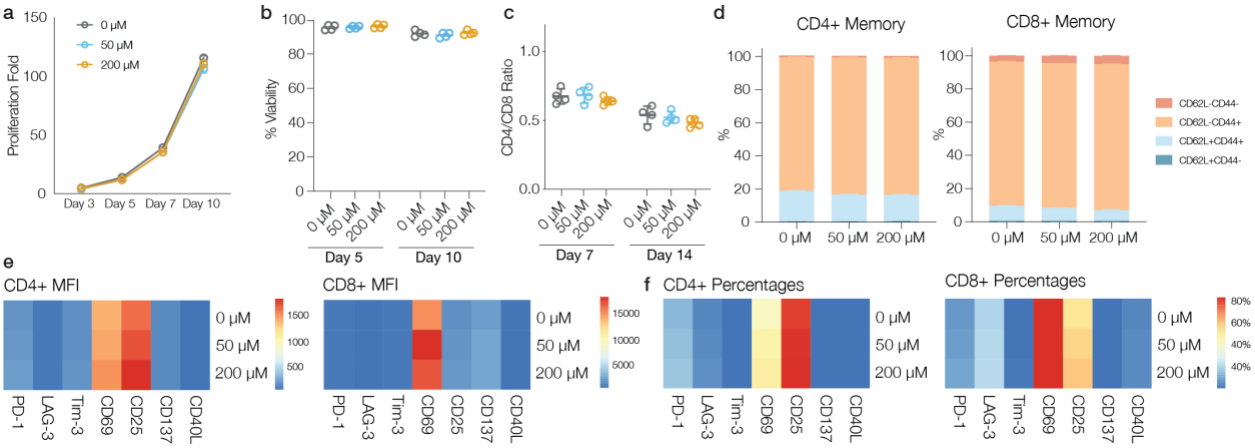

Figure S1. Azido-sugar nanoparticles label T cells without affecting T cell function and phenotype. **a,** Cell count, **(b)** viability, and **(c)** CD4/CD8 ratios in T cells incubated in various concentrations of G400 NP over time. **d,** Percentages of T cell populations expressing markers indicative of different memory phenotypes on day 10. **e,** MFI of and **(f)** percentage of positive cells for various activation and exhaustion markers T cells incubated in various concentrations of G400 NP on day 10 of culture. (n=3)

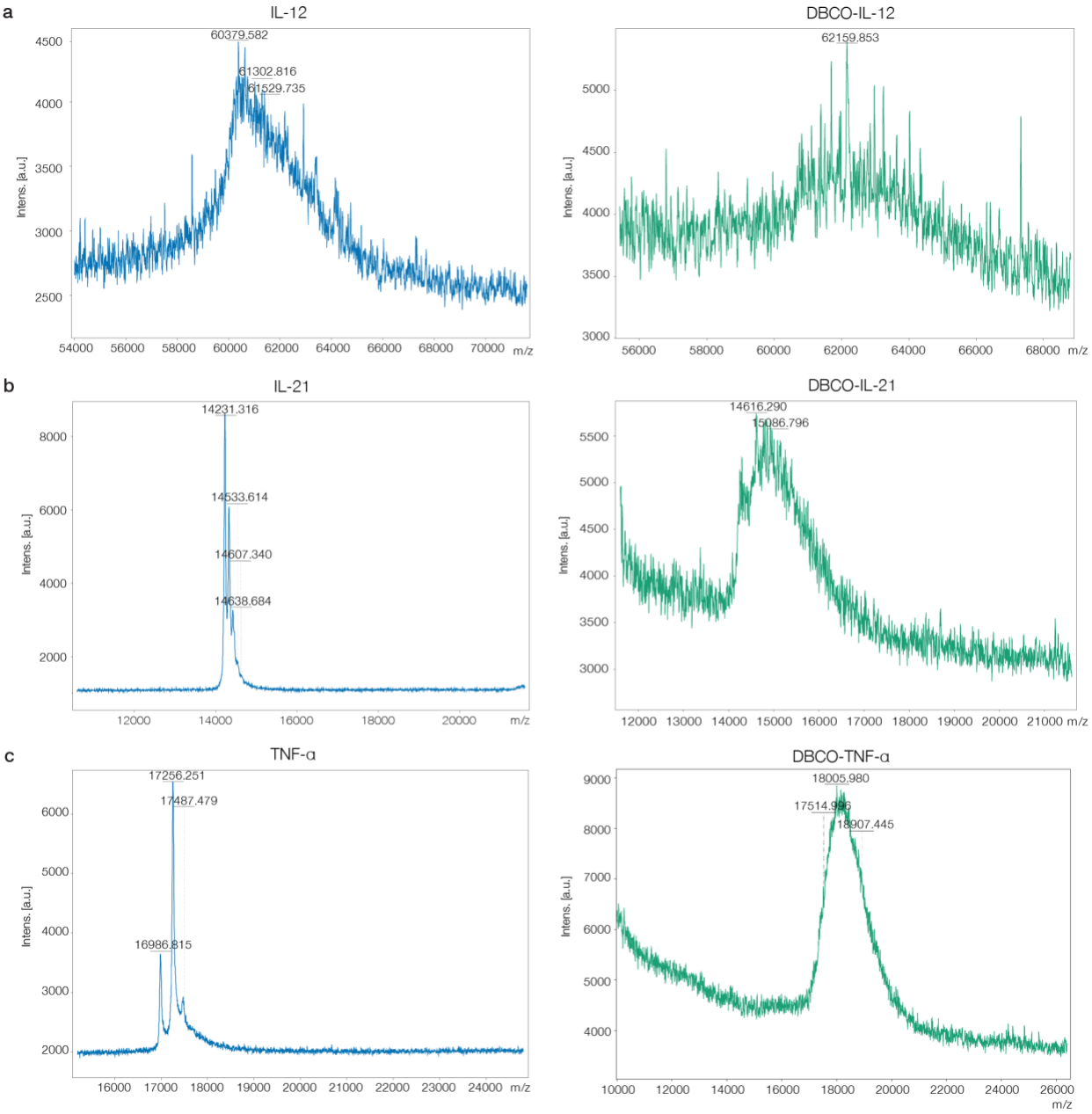

Figure S2. MALDI-TOF spectrum for unmodified and DBCO-modified cytokines. a, IL-12, **(b)** IL-21, and **(c)** TNF-α. Each cytokine molecule has an average of 2-3 conjugated DBCO groups.

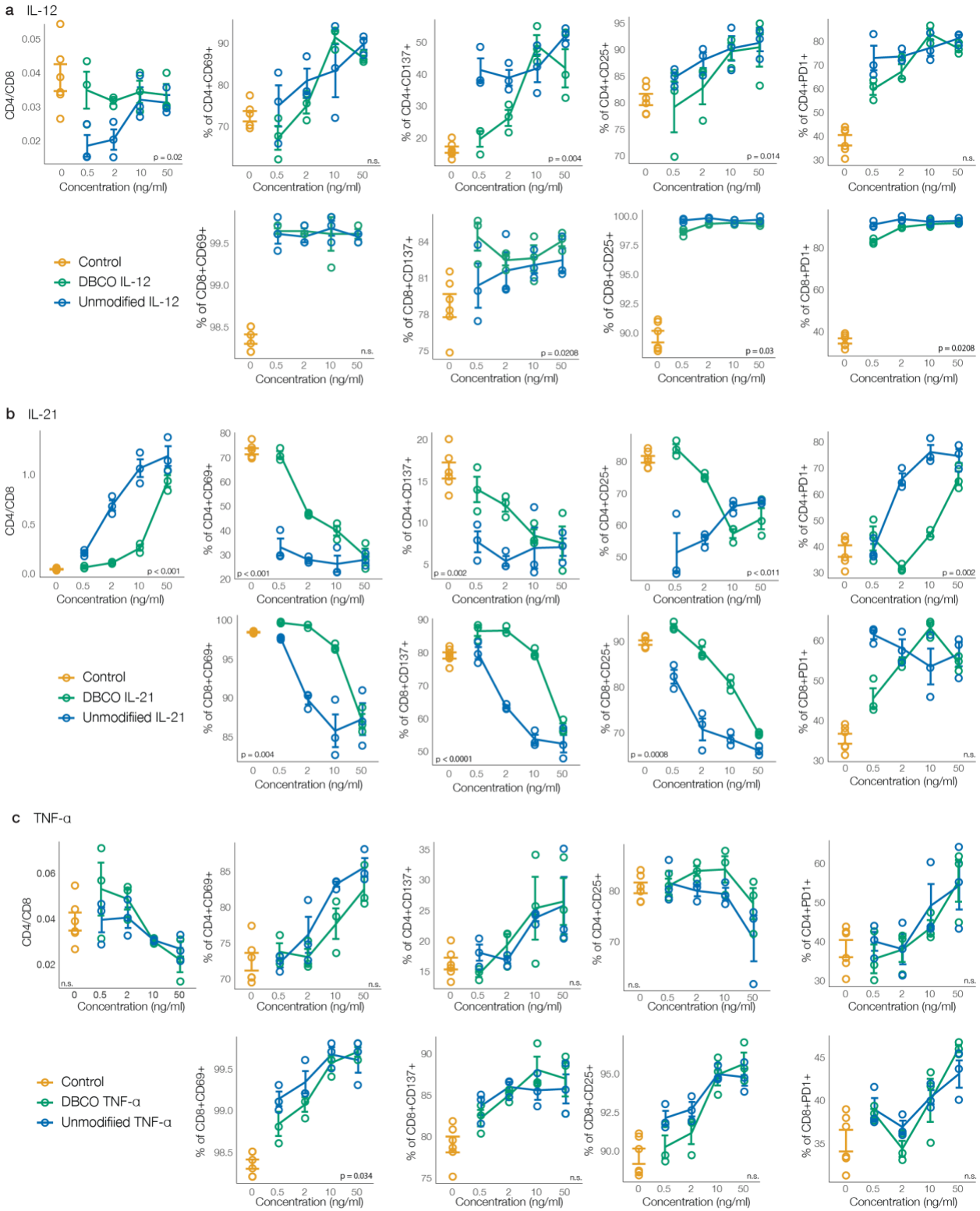

Figure S3. **DBCO-modified cytokines exhibit slightly lower bioreactivity compared with unmodified counterparts.** Representative phenotyping data of showing CD4/CD8 ratio, and percentages of positive cells for various activation and exhaustion markers for T cells treated with various concentrations of soluble **(a)** DBCO- and unmodified- IL-12, **(b)** DBCO- and unmodified- IL-21, **(c)** DBCO- and unmodified- TNF-α. (n=3, two-way ANOVA test)

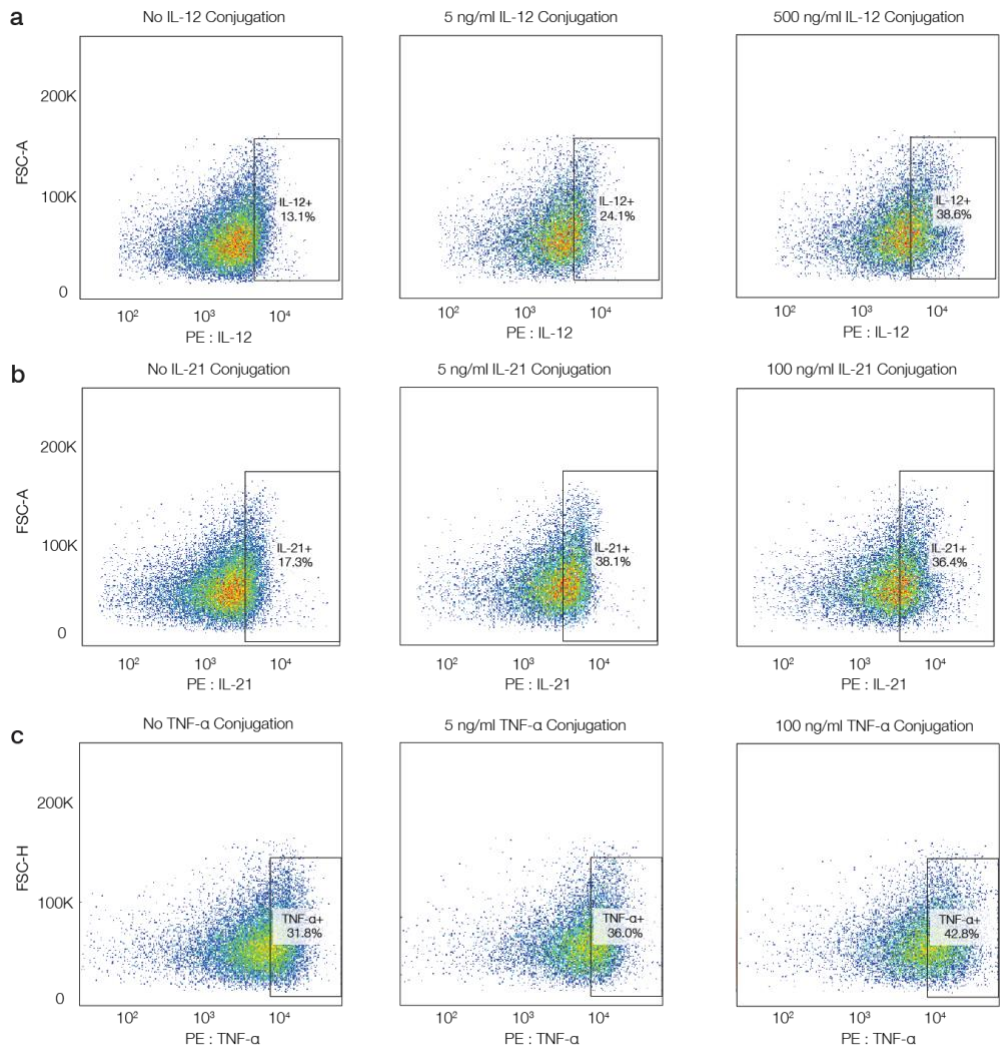

Figure S4. DBCO-cytokine could be conjugated onto G400 NP treated T cells. Flow cytometry data showing percentage of T cells with positive staining for **(a)** IL-12, **(b)** IL-21, and **(c)** TNF-α when conjugated with various concentrations of cytokines. Representative groups are T cells conjugated with DBCO-cytokine ("NP + Conjugated cytokine"; green), treated with continuously soluble cytokine ("NP + Continuous cytokine"; blue), or temporarily exposed to cytokine for duration of reaction time ("NP + Transient cytokine", purple).

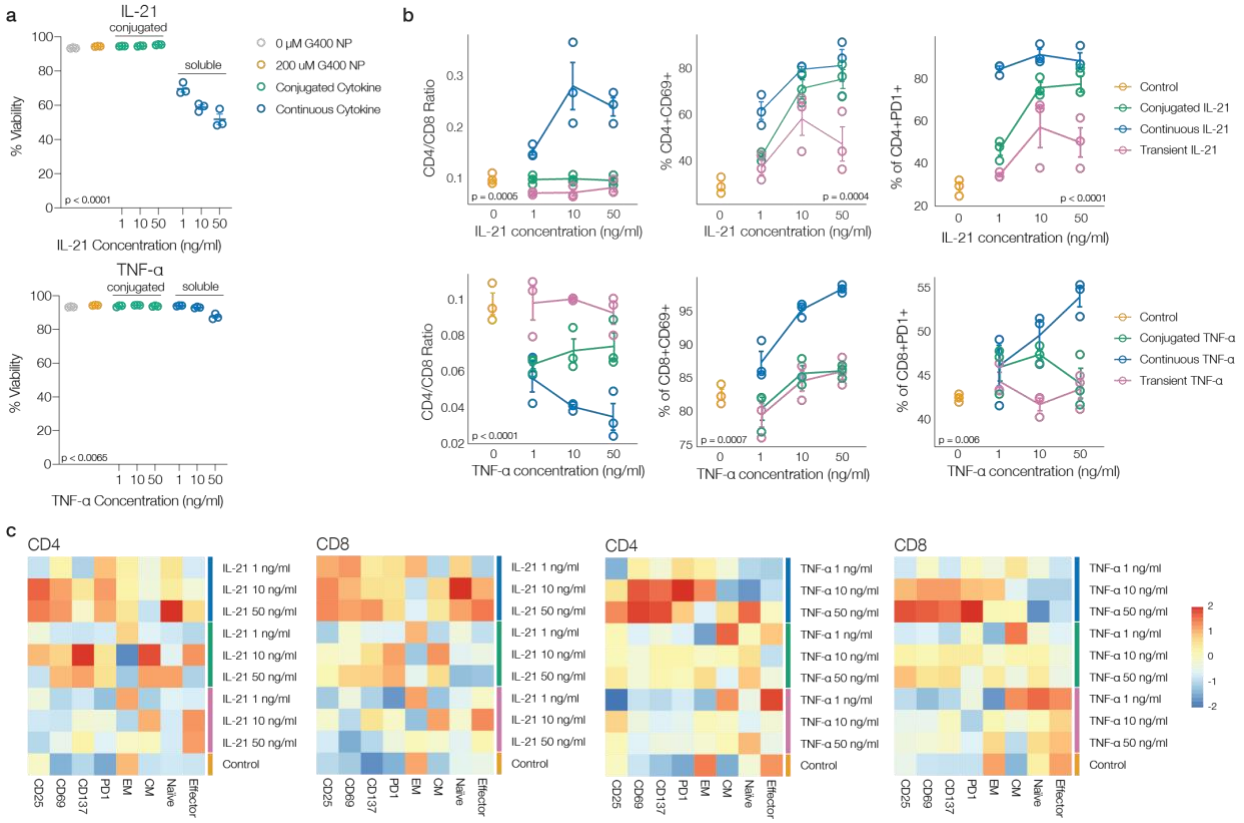

Figure S5. Conjugating cytokines onto T cells affects T cell phenotype without inducing cytotoxicity. a, Viability data for T cells receiving no IL-21 or TNF-α, conjugated with DBCO-IL-21 or DBCO-TNF-α, and presented with soluble IL-21 or TNF-α (no DBCO label) in media in *in vitro* culture (n=3, two-way ANOVA test). **b,** Representative phenotyping data and **(c)** Heatmap data showing memory phenotype markers and activation and exhaustion markers, for T cells conjugated with DBCO-cytokine ("Conjugated cytokine"; green), treated with soluble cytokine ("Continuous IL-12"; blue), or temporarily exposed to cytokine for duration of reaction time ("Transient cytokine", purple) at different cytokine concentrations (n=3, two-way ANOVA test). **d,** Cytolytic activities of Pmel-1 T cells conjugated with DBCO-cytokine, treated with soluble cytokine, or temporarily exposed to cytokine against B16-F10 tumor cells (n=3).

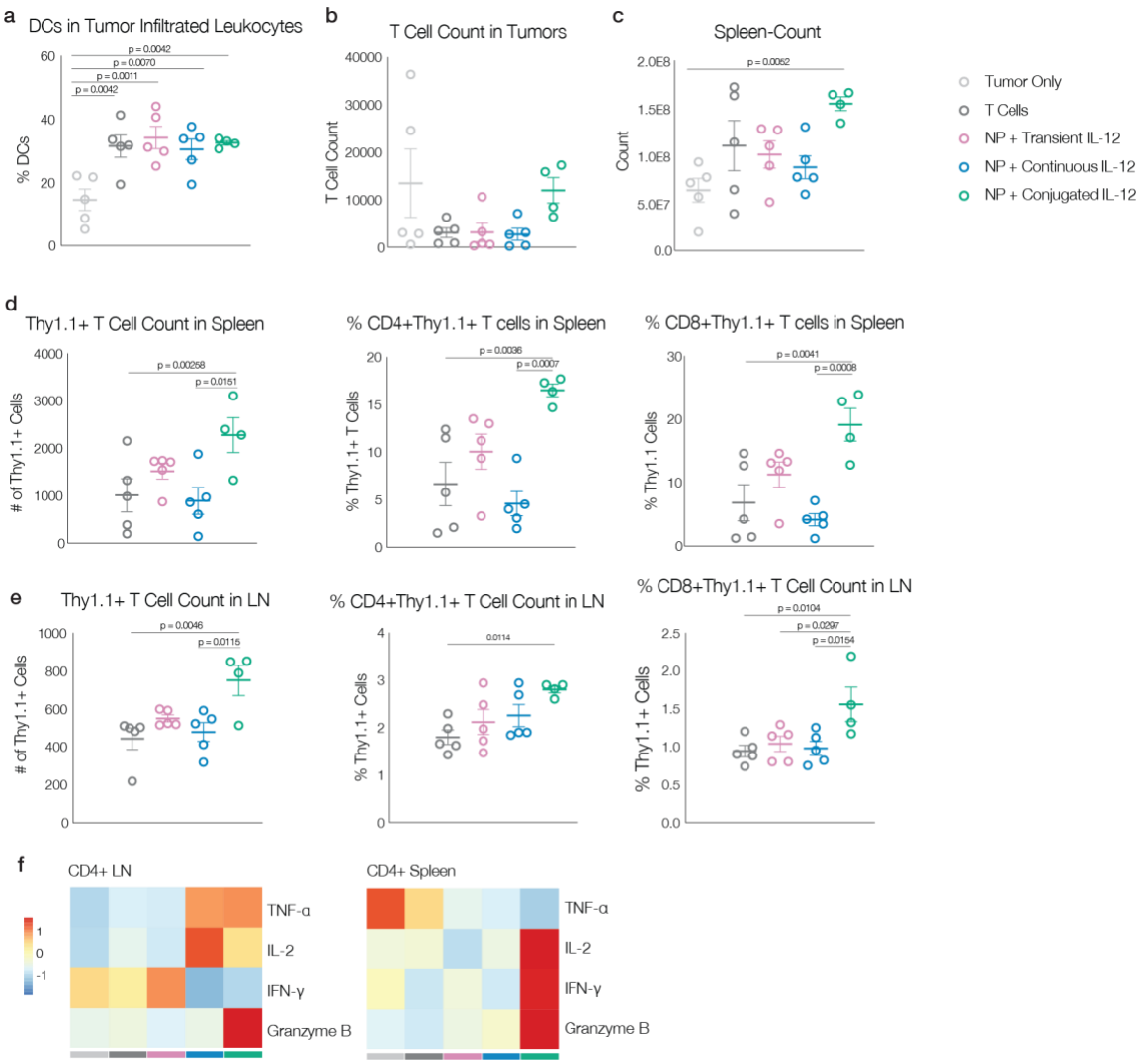

Figure S6. Conjugating IL-12 on T cell surfaces increases T cell infiltration in solid tumors, increases antigen presentation, and promotes antigen spreading. a, percent of DC cells in tumor infiltrated leukocytes (n=4-5). **b,** total T cell count in tumors (n=4-5). **c,** total number of leukocytes in spleen (n=4-5). **d,** total number of Thy1.1+ Pmel-1 T cells, and percent of CD4+ and CD8+ Thy1.1+ T cells in spleen (n=4-5). **e,** total number of Thy1.1+ Pmel-1 T cells, and percent of CD4+ and CD8+ Thy1.1+ T cells in tumor draining lymph nodes (n=4-5). **f,** heatmap of average expression level of Th1 cytokines in CD4+ T cells in lymph nodes and spleen after isolation and *ex vivo* antigen stimulation (n=5). Animals were untreated ("Tumor only"; light grey), treated with CAR-T cells without labeling but without IL-12 conjugation ("CAR-T"; dark grey), treated with CAR-T cells metabolically labeled and transiently exposed to non-DBCO conjugated IL-12 prior to transfer ("NP + transient IL-12"; purple), treated with CAR-T cells that were labeled and transferred with the same quantify of soluble IL-12 (not DBCO conjugated) as was conjugated to CAR-T cells ("NP + continuous IL-12"; blue), or treated with CAR-T cells that were metabolically labeled and then conjugated with IL-12 prior to transfer ("NP + conjugated IL-12"; green). (Statistical analyses performed with one-way ANOVA and Tukey's test)

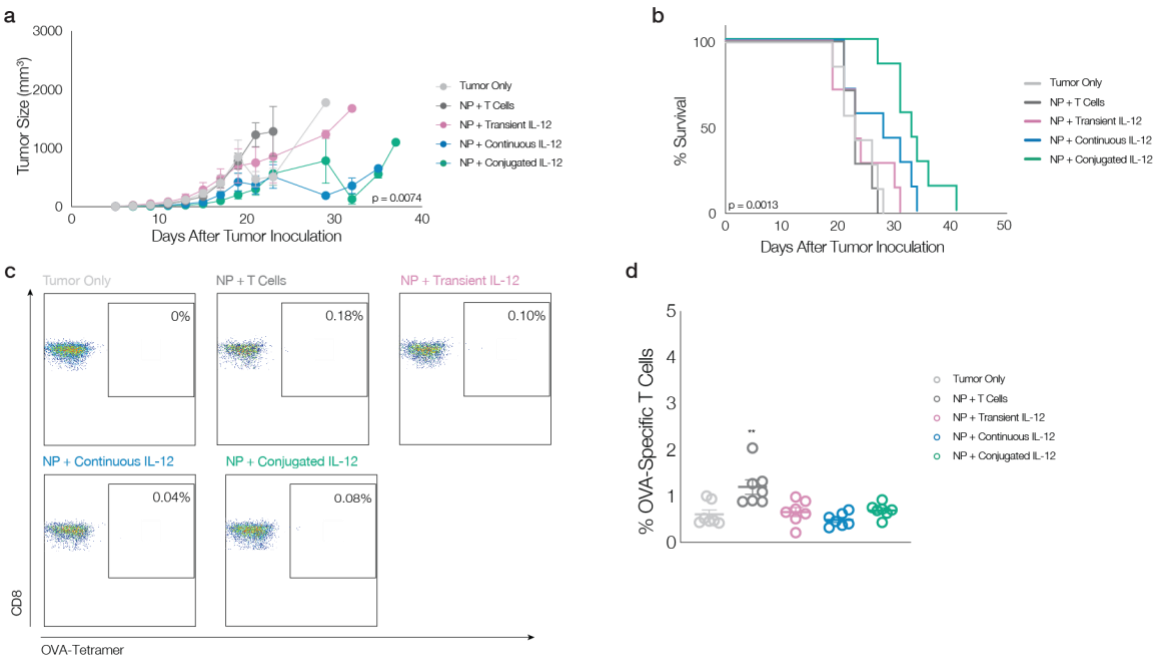

Figure S7. IL-12 conjugation onto T cells in B16-OVA model. a, Mouse B16-OVA tumor growth (n=7, two way-ANOVA test) and **(b)** survival (n=7, Mantel-Cox test). **c,** Representative flow cytometry plots and **(d)** summary of flow cytometry data for OVA-specific CD8+ T cells on Day 2 after T cell adoptive transfer (n=7). Animals were untreated (“Tumor only”; light grey), treated with T cells without labeling and IL-12 conjugation (“T cells”; dark grey), treated with T cells metabolically labeled and transiently exposed to non-DBCO conjugated IL-12 prior to transfer (“NP + transient IL-12”; purple), treated with T cells that were labeled and transferred with the same quantify of soluble IL-12 (not DBCO conjugated) as was conjugated to T cells (“NP + continuous IL-12”; blue), or treated with T cells that were metabolically labeled and then conjugated with IL-12 prior to transfer (“NP + conjugated IL-12”; green).

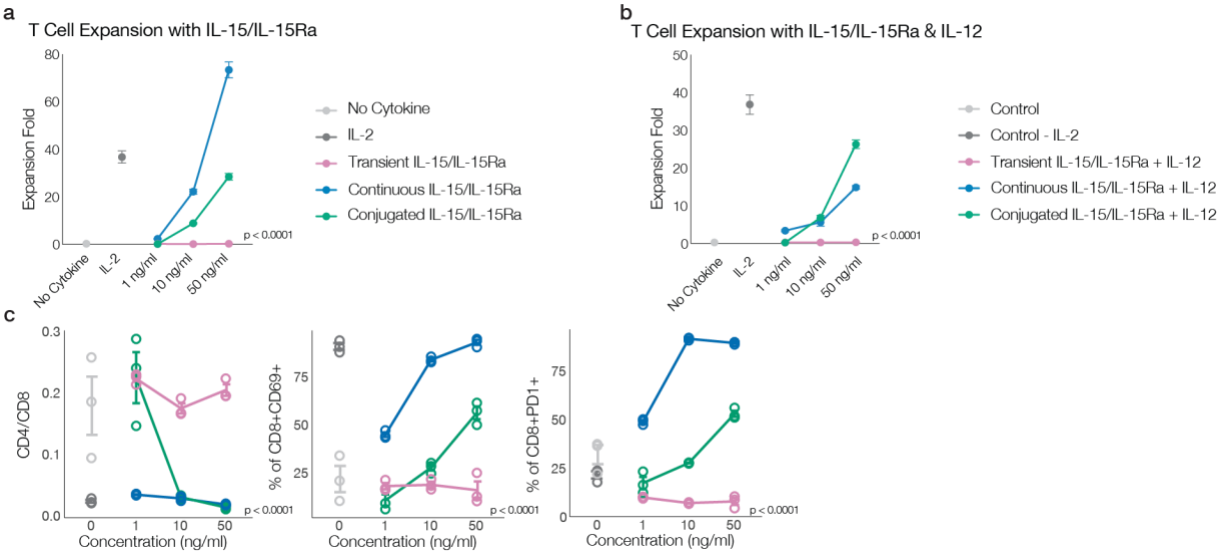

Figure S8. Conjugating multiple DBCO-cytokines achieves synergistic effect on T cells. a, Expansion data for T cells conjugated with DBCO-IL-15/IL-15Rα (“Conjugated IL-15/IL-15Rα”; green), treated with continuously soluble IL-15/IL-15Rα (“Continuous IL-15/IL-15Rα”; blue), or temporarily exposed to IL-15/IL-15Rα for duration of reaction time (“Transient IL-15/IL-15Rα”, purple) at different IL-15/IL-15Rα concentrations. **b,** Expansion data for T cells conjugated with both DBCO-IL-12 and DBCO-IL-15/IL-15Ra (“Conjugated IL-15/IL-15Rα + IL-12”; green), treated with continuously soluble IL-12 and IL-15/IL-15Ra (“Continuous IL-15/IL-15Rα + IL-12”; blue), or temporarily exposed to IL-12 and IL-15/IL-15Ra for duration of reaction time (“Transient IL-15/IL-15Rα + IL-12”, purple) at different cytokine concentrations. **c,** Representative phenotyping data showing activation and exhaustion markers, for T cells conjugated with both DBCO-IL-12 and DBCO-IL-15/IL-15Ra, treated with soluble IL-12 and IL-15/IL-15Ra, or temporarily exposed to IL-12 and IL-15/IL-15Ra duration of reaction time at different cytokine concentrations. (n=3)

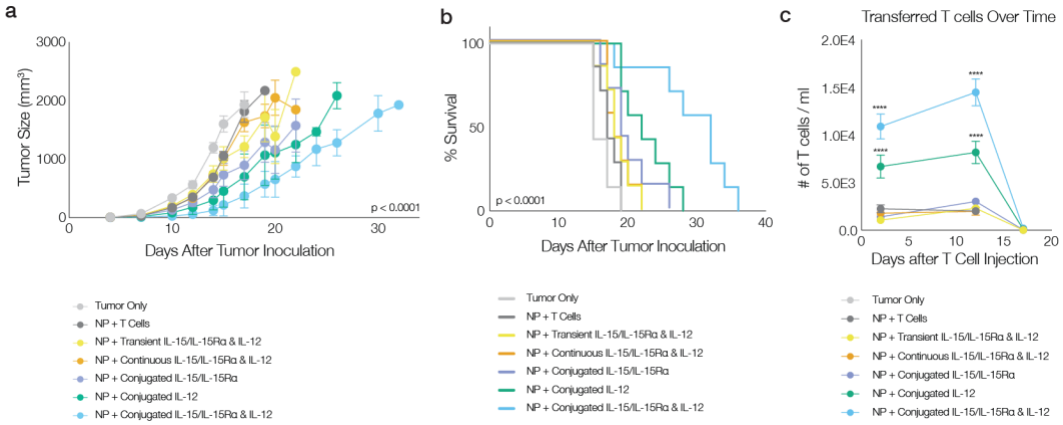

Figure S9. Conjugating multiple DBCO-cytokines significantly slow tumor growth as compared to single cytokine conjugation. a, mouse tumor growth (n=7, two-way ANOVA test) and **(b)** survival data (n=7, Mantel-Cox test) in B16-F10 melanoma model. **c,** number of Thy1.1+ CD8+ Pmel-1 T cells per 1 mL of blood over therapeutic study (n=7). Animals were untreated (“Tumor only”; light grey), treated with T cells without labeling or cytokine conjugation (“T cells”; dark grey), treated with T cells metabolically labeled and transiently exposed to non-DBCO conjugated IL-15/IL-15Rα and IL-12 (“NP + transient IL-15/IL-15Rα & IL-12”; yellow), treated with CAR-T cells that were labeled and transferred with the same quantify of soluble IL-15/IL-15Rα andIL-12 (not DBCO conjugated) as was conjugated to T cells (“NP + continuous IL-15/IL-15Rα + IL-12”;orange), treated with T cells that were metabolically labeled and conjugated with IL-15/IL-15Rα (“NP + conjugated IL-15/IL-15Rα”; orchid), treated with T cells that were metabolically labeled and conjugated with IL-12 (“NP + conjugated IL-12”, green), treated with T cells that were metabolically labeled and conjugated with IL-15/IL-15Rα and IL-12 (“NP + conjugated IL-15/IL-15Rα & IL-12”, sky blue).