Supporting Information

Multi-parameter longitudinal imaging of immune cell activity in chimeric antigen receptor T-cell and checkpoint blockade therapies

Jinghang Xie†, Fadi El Rami†, Kaixiang Zhou†, Federico Simonetta2, Zixin Chen3, Xianchuang Zheng1, Min Chen1, Preethi B. Balakrishnan1, Sheng-Yao Dai1, Surya Murty1,4, Israt S. Alam1, Jeanette Baker2, Robert S. Negrin2, Sanjiv S. Gambhir1,4,5, Jianghong Rao1,3*

1Department of Radiology, Molecular Imaging Program at Stanford, Stanford University School of Medicine, Stanford CA 94305 (USA)
2Division of Blood and Marrow Transplantation, Department of Medicine, Stanford University Medical Center, Stanford, CA 94305 (USA)
3Department of Chemistry, Stanford University, Stanford, CA 94305 (USA)
4Department of Bioengineering, Stanford University, Stanford CA 94305 (USA)
5Department of Materials Science & Engineering, Stanford University, Stanford CA 94305 (USA)

†These authors contributed equally to this work.
*Corresponding author. E-mail: jrao@stanford.edu
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Materials and Methods

1. General information – chemistry

Mouse gzmB substrate IEFD (Iso-Glu-Phe-Asp) with N-terminal acetylation was customized from GenScript USA (Piscataway, NJ). All chemicals were obtained from commercial sources unless otherwise stated. Reactions were monitored by thin layer chromatography (TLC) on 0.25 mm silica gel 60F plates. The $^1$H and $^{13}$C NMR spectra were acquired on Inova 500 MHz nuclear magnetic resonance (NMR) spectrometers at Stanford University. Data for $^1$H NMR spectra are reported as follows: chemical shifts are reported as $\delta$ in units of parts per million (ppm). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), quint (quintet), m (multiplet), or br (broadened); coupling constants are reported as a J value in Hertz (Hz); the number of protons (n) for a given resonance is indicated nH, and based on the spectral integration values. HRMS samples were performed on ESI-MS at the Waters Acquity UPLC and Thermo Exactive Orbitrap mass spectrometer at the Vincent Coates Foundation Mass Spectrometry Laboratory, Stanford University Mass Spectrometry. High-performance liquid chromatography (HPLC) was performed on a Dionex Ultimate 300 HPLC System (Thermo Scientific) equipped with a GP50 gradient pump and an in-line diode array UV-Vis detector. Reverse-phase C18 (Phenomenex, 5 μm, 4.6 x 250 mm or Dionex, 5 μm, 21.2 x 250 mm) columns were used with acetonitrile/water gradient mobile phase containing 0.1% trifluoroacetic acid (at a flow rate of 1 or 12 mL/min for analysis or purification respectively). TEM was performed on a JEOL JEM1400 transmission electron microscope. DLS and Zeta potentials were measured on Malvern ZetaSizer.

2. Compound syntheses and characterizations

Synthesis of G-SNAT precursor is shown in Fig. S1.

Synthesis of G-SNAT and G-SNAT-Cy5 is shown in Fig. S2.

(45,10R,13S,16S,19S)-19-((25,3S)-2-acetamido-3-methylpentanamido)-16-benzyl-13-(carboxymethyl)-1-(4-((2-(cyano(pyrimidin-5-yl)oxy)propyl)carbamoyl)phenyl)-10-((ethylsulfinothioyl)methyl)-3,6,9,12,15,18-hexaazadocosan-22-oic acid (G-SNAT)

(i) To a mixture of Ac-IEFD-OH (0.05 mmol, 33.8 mg), HBTU (0.75 mmol, 28.4 mg) and compound 6 (0.05 mmol, 31.3 mg) was added DMF (15 mL), followed by N, N-Diisopropylethylamine (DIPEA, 0.25 mmol, 32.4 mg). The resulting solution was stirred for 2.5 h at r.t., then concentrated under high vacuum to remove DMF. The resulting residue was washed with water and brine, dried over anhydrous Na$_2$SO$_4$, filtered, and concentrated.

(ii) The remaining residue was added with a solution of CF$_3$COOH: DCM: TIPS = 1: 1: 0.05 (10 mL) and continued to stir at r.t. for another 2 h. After concentration, the final product G-SNAT was purified by preparative-HPLC to afford a white powder (10.0 mg, 17.0%). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.10 (s, 1H), 8.60 (t, $J$ = 6.0 Hz, 1H), 8.50 (t, $J$ = 5.7 Hz, 1H), 8.39 (d, $J$ = 7.7 Hz, 1H), 8.18 – 8.12 (m, 3H), 7.94 (dd, $J$ = 17.9, 8.0 Hz, 2H), 7.85 (d, $J$ = 8.0 Hz, 1H), 7.77 (d, $J$ = 8.2 Hz, 2H), 7.33 (d, $J$ = 8.1 Hz, 2H), 7.22 (d, $J$ = 4.3 Hz, 4H), 7.15 (dt, $J$ = 8.7, 4.2 Hz, 1H), 4.60 (q, $J$ = 7.2 Hz, 1H), 4.53 (d, $J$ = 8.6 Hz, 1H), 4.51 – 4.45 (m, 2H), 4.45 (d, $J$ = 6.7 Hz, 2H), 4.38 (dd, $J$ = 15.7, 6.1 Hz, 1H), 4.30 (dd, $J$ = 15.8, 5.8 Hz, 1H), 4.19 (q, $J$ = 7.9 Hz, 1H), 4.10 (t, $J$ = 7.8 Hz, 1H), 3.77 (qd, $J$ = 16.7, 5.7 Hz, 2H), 3.11 (dd, $J$ = 13.5, 4.8 Hz, 1H), 3.03 (dd, $J$ = 14.0, 4.8 Hz, 1H).
4.0 Hz, 1H), 2.94 – 2.85 (m, 2H), 2.82 – 2.65 (m, 4H), 2.64 – 2.56 (m, 1H), 2.53 (d, J = 7.4 Hz, 2H), 2.22 – 1.97 (m, 4H), 1.85 (s, 3H), 1.81 (s, 1H), 1.68 (dd, J = 15.6, 8.5 Hz, 2H), 1.38 (s, 1H), 1.21 (q, J = 6.5 Hz, 3H), 1.06 (dt, J = 14.7, 7.6 Hz, 1H), 0.82 – 0.73 (m, 6H).

13C NMR (126 MHz, DMSO-d6) δ 174.0, 171.8, 171.3, 171.1, 170.9, 170.7, 170.0, 169.7, 169.5, 168.5, 166.1, 165.1, 163.4, 142.2, 137.6, 133.0, 129.1, 128.0, 127.1, 126.8, 126.2, 115.6, 102.2, 80.5, 73.2, 66.4, 57.1, 53.6, 52.4, 51.9, 51.7, 42.2, 41.9, 40.5, 40.0, 39.9, 39.9, 39.8, 39.7, 39.5, 39.4, 39.2, 39.0, 37.6, 36.2, 36.0, 31.7, 30.1, 28.4, 27.2, 24.5, 22.5, 21.8, 15.4, 14.3, 11.0. MS: m/z calcd. for

C_{54}H_{68}N_{12}O_{14}S_{2} 1172.4; found 1171.3, [M + H]^+.

Compound G-SNAT (6.1 µmol, 7.1 mg) and sulfo-Cy5-azide (6.5 µmol, 5.0 mg) was dissolved in DMSO (200 µL) and 0.1 M HEPES solution (800 µL), then CuSO_4 (100 µL of 0.1 M stock solution in H_2O), (BimC_4A)_3 (100 µL of 30 mM stock solution in H_2O) and sodium ascorbate (100 µL of 1 M stock solution in H_2O, freshly prepared) were added to the mixture. After stirring at r.t. for 1 h, the mixture was directly purified by preparative-HPLC to final product G-SNAT-Cy5 as a blue powder (3.2 mg, 28.1%). MS: m/z calcd for C_{89}H_{111}N_{18}O_{21}S_{4} 1895.7; found 947.8, [M + 2H]^2+; HRMS (ESI/Q-TOF): [M + 2H]^2+ m/z calcd. for C_{89}H_{111}N_{18}O_{21}S_{4} 949.3639; found 949.3642.
3. General information – biology

Recombinant mouse granzyme B protein (C-6His, C765) was purchased from Novoprotein Scientific (Summit, NJ). Recombinant mouse active cathepsin C/DPPl Protein (2336CY010) was purchased from R&D systems (Minneapolis, MN). Recombinant human caspase-3 protein (235417) was purchased from Sigma-Aldrich (St Louis, MO). Granzyme B inhibitor (368055) was purchased from EMD Millipore (Burlington, MA). Rat anti-granzyme B mouse monoclonal antibody (16G6) was from Invitrogen (Carlsbad, CA). Anti-mouse PD-1 (clone-RMP1-14) and anti-mouse CTLA-4 (clone-9D9) were purchased from BioXcell (Lebanon, NH). IRDye 680RD goat anti-rat IgG, IRDye 680RD donkey anti-mouse IgG and IRDye 800CW donkey anti-rabbit secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). FITC conjugated anti-mouse CD3 (17A2) and Alexa Fluor 594 anti-mouse CD19 (6D5) antibodies were purchased from Biolegend (San Diego, CA). Rabbit anti-β Actin polyclonal antibody (PA1-46296) was from Invitrogen. D-luciferin, potassium salt was purchased from Gold Biotechnology (St Louis, MO). Cyanine 5 azide was purchased from Lumiprobe (Hunt Valley, Maryland). RIPA buffer, Pierce BCA protein assay reagents and NuPAGE 4-12% Bis-Tris protein gels were purchased from Thermo Fisher Scientific (Waltham, MA). Microtube pestles were purchased from USA Scientific (Ocala, FL). Mouse serum was purchased from Sigma-Aldrich (St Louis, MO). Hoechst 33342 was purchased from Sigma-Aldrich. High-precision cover glass 22 x 22 mm with thickness 170 μm ± 5 μm (0107052) was purchased from Marienfeld-Superior (Lauda-Königshofen, Germany). Superfrost Plus microscope slides (12-550-15) were purchased from Fisher Scientific. SlowFade Diamond antifade mountant (S36963) was purchased from Invitrogen. Western blots were imaged using a LI-COR Odyssey imaging system.

4. Cell culture

A20 (TIB-208) murine B lymphocyte cell line was obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate. Culture was maintained in 37°C with 5% CO2. The modified A20 FLuc+/YFP/neo was generated as previously reported1. Cells were grown in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (ThermoScientific), 2 mM L-glutamine (Sigma) and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) at 37 °C in 5% CO2 atmosphere. B16 melanoma cell line was obtained from ATCC. SB28, a murine glioblastoma cell line was generously provided from Dr. Hideho Okada (University of California San Francisco, San Francisco, CA). Both cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibacterial/antimycotic solution (Catalog#15240112, ThermoFisher). B16 and SB28 lines were engineered to express murine CD19-TurboRFP-Rluc and GD2-TurboRFP, respectively, using lentiviral transduction followed by three rounds of cell sorting for the highest 2.5% of TurboRFP expressers. CT26 murine colon carcinoma cell line was obtained from ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Culture was maintained in 37°C with 5% CO2.
All cell lines were routinely tested for mycoplasma contamination (MycoAlert Mycoplasma Detection Kit purchased from Lonza), with authentication performed at Stanford Functional Genomics Facility for Short Tandem Repeat (STR) profiling.

5. Viral vectors

MSGV retroviral vectors encoding second generation CARs, CD19-28ζ or GD2-4-1BBζ CAR\(^2\), were used for construction of CD19(murine)-targeted CART cells or GD2-targeted CAR T cells. 293GP producer cell lines, provided as a gift from Dr. Crystal Mackall (Stanford University, Stanford, CA), produced retroviral supernatant harboring CD19-28ζ or GD2-4-1BBζ CAR vectors. Supernatant was collected after 72 hours of 293GP culture, centrifuged to discard cell debris, and stored at -80°C for 6 months.

For retroviral transduction, non-tissue culture treated 6-well plates (Catalog# 3736, Corning, NY) were coated with Retronectin (Takara, Japan) diluted in PBS (24 µg/ml) for 16 hours at 4°C. Plates were blocked with 2% BSA in PBS for 30 minutes and then discarded. Frozen supernatants were thawed on ice and 2 ml supernatant was mixed with 1 ml T cell culture medium and added to each well, followed by centrifugation for 3 hours at 3200 rpm at 32°C. Supernatants were discarded and plates were ready for T cell addition.

6. Generation of CD19-28ζ and GD2-4-1BBζ CAR T cells

Splenocytes were harvested from 8–10 weeks old, female BALB/c mice (Charles River Laboratories, Wilmington, MA) or 6–8 weeks old, female C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME) and processed in 1× PBS (Invitrogen) supplemented with 2% FBS (Invitrogen) into single-cell suspensions. CD8+ T cells were prepared using EasySep™ Mouse CD8+ T Cell Isolation Kit (Catalog# 19853, Stemcell Technologies, Vancouver, Canada). Total T cells were prepared using EasySep™ Mouse Total T Cell Isolation Kit (Catalog# 19851, Stemcell Technologies, Vancouver, Canada), as recommended by the manufacturer. In brief, cells were blocked with normal rat serum, stained with Isolation cocktail antibodies, mixed with Streptavidin RapidSpheres, and purified cells were isolated manually using MACS EasySep™ magnetic column (Catalog #18000, Stemcell Technologies, Vancouver, Canada. This purification protocol yielded >90% purity.

CD19-28ζ and GD2-4-1BBζ CAR-T cells were genetically engineered from CD8+ T cells and total T cells, respectively, using retroviral transduction\(^3\). The enriched T cell fractions were activated with magnetic beads coated with agonistic anti-CD3 and CD28 antibodies (Catalog# 11456D, ThermoFisher, Waltham, MA) at 1 : 1 (beads : cells) ratio in T cell medium (RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% antibacterial/antimycotic solution, 10 U/ml recombinant murine IL-2 (Peprotech, NJ), 10 U/ml recombinant murine IL-7 (Peprotech, NJ), and 0.05 mM 2-mercaptoethanol). After 24 hours, 1 x 10\(^6\) activated T cells were transduced with retroviruses bound to retronectin-coated tissue culture plates for 3 days, after which beads were removed. Transduced and untransduced T cells were maintained at 1x10\(^6\) cells per milliliter in T cell medium and analyzed using flow cytometric analysis. For mice studies, T cells were transferred three days after removal of beads and viral
supernatant. CAR T cells generated from BALB/c mice were used to challenge A20 cells, while those produced from C57Bl/6J were used to challenge B16 cells.

7. Flow cytometry

For T cell sorting, cells were harvested, washed twice with 1× PBS, and re-suspended in cold PBS containing 2% FBS (at a density of 1 × 10^6 cells/100µl). Subsequently, primary labelled antibodies or Pierce™ Recombinant Protein L (catalog #21189, ThermoFisher) were added into the cell suspension according to the manufacturer’s instructions and incubated for 20 minutes at 4 °C in the dark. Immediately after the incubation, the cells were washed thrice with ice cold PBS. For the protein L treated cells, APC-labelled streptavidin was added to cells and incubated for 15 minutes at 4 °C in the dark. Flow cytometry was performed using a BD LSRII and FlowJo Software (TreeStar) for analysis. Gating strategies for detecting the percentages of naïve, CD8+ T cells and CAR-T cells were analyzed using the following antibodies: anti-CD3 (clone: 17A2, Brilliant Violet 421), anti-CD8 (clone: 53-6.7, FITC or clone: YTS156.7.7, PE/Dazzle 594), anti-CD44 (clone: IM7, PE), anti-CD19 (PE/APC/FITC), anti-CD62L (clone: MEL-14, Alexa Fluor 700), anti-CTLA4 (clone UC10-4B9, PE), anti-PD1 (clone: 29F.1A12, APC/Fire 750), and streptavidin (APC). Dead cells were excluded by using Zombie NIR™ Fixable Viability kit (catalog# 423105). The antibodies used for flow cytometric analysis were obtained from Biolegend.

For analysis of CAR or CD8+ T cells treated A20 cells as suspension in the presence of G-SNAT-Cy5, cells were centrifuged gently at 300 g for 10 min, washed with cold PBS once, then analyzed with a 4-laser, 12-color DxP12 Cytek upgrade (Becton Dickinson, Cytek Biosciences) and evaluated according to their size (FSC, Front Scatter) on a linear scale. For Cy5, cells were excited by 640 nm later, filtered by 655 nm, LP (Long Pass filter) and 661/716 nm, BP (Band Pass filter). For FITC α-CD3 antibody, cells were excited by 488 nm later, filtered by 560 nm, SP (Short Pass filter) and 525/550 nm, BP. For Alexa Fluor 594 conjugated α-CD19 antibody, cells were excited by 561 nm later, filtered by 600 nm, SP (Short Pass filter) and 590/620 nm, BP. Flow cytometry data analysis and 3D dimensional projection was done using FlowJo V10 software. The MFI (mean fluorescence intensity) was collected and plotted.

8. Cellular Uptake Assay

For G-SNAT-Cy5 internalization assay, untransduced activated CD8+ T cells were pre-incubated at 4 or 37 °C for 30 min then treated with PBS or 5 µM G-SNAT-Cy5 in culture medium for 2.5 h at 4 or 37 °C. Cells were washed twice with PBS then stained with Zombie Aqua viability dye (423101, Biolegend) according to manufacturer protocol. Cells were washed twice and fixed in 4% paraformaldehyde at room temperature for 30 min before analysis with flow cytometry. The MFI-Cy5 of viable cells were acquired and plotted with flowjo 10.6.

9. Western blot analysis of cells and tumors

Cells were trypsinized, pelleted and washed 3 times with ice cold PBS and lysed in RIPA buffer. Tumor tissues were cut into small pieces and grinded in 1.5 mL Eppendorf tubes with microtube pestles for lysis in RIPA buffer. Protein concentration of centrifuged whole lysate were determined with BCA assay. Tumor lysates were loaded in NuPAGE 4-12% Bis-Tris protein gels for
electrophoresis at 200V for 90 min. Wet transfer were performed using a Bio-Rad transfer kit at 300 mA for 90 min. The transferred nitrocellulose membrane was blocked in PBS containing 5% BSA and 0.1% Tween-20 for 1 h. Primary antibody incubation was performed in the blocking buffer overnight at 4 °C in recommended concentration. The membrane was then washed with PBS containing 0.1% Tween-20 for 5 min, four times. IRdye conjugated secondary antibody incubation was performed in the blocking buffer for 2 h at room temperature. After washing four times with PBS containing 0.1% Tween-20, membranes were analyzed in a LI-COR Odyssey imaging system.

10. Granzyme B-mediated macrocyclization in vitro

G-SNAT (10 μM, 10 μL of 1 mM stock in DMSO) was diluted in reaction buffer containing 50 mM Tris at pH 7.5 with or without 1 mM TCEP (2 μL of 500 mM stock in water) and 1 μg recombinant mouse gzm B in 1 mL final concentration. Reactions were performed at 37 °C overnight and monitored by HPLC. For kinetic study, G-SNAT (10 μM) was incubated with equal amount (100 U) of recombinant mouse GzmB (0.05 μg/ml) in Tris buffer and human caspase-3 in a buffer containing 50mM HEPES, 100 mM NaCl, 1 mM EDTA and 10% glycerol for up to 8 hours.

11. Quantitative real-time PCR

Total RNA was extracted from 1 million of each T cell population tested using RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. cDNA synthesis was conducted using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) as follows: 1 μg sample RNA mixed with 4 μL of 5x iScript reaction mix and 1μL of iScript reverse transcriptase in total volume of 20 μL. Reaction protocol as follows: 5 min at 25 °C for priming, 20 min at 46 °C for reverse transcription, 1 min at 95 °C for inactivation of reverse transcription. PrimePCR™ FAM-conjugated Probe Assays (Bio-Rad, Hercules, CA) for murine granzyme B (Assay ID: qMmuCEP0053372) and GAPDH (Assay ID: qMmuCEP0039581) were used with Sso Advanced Universal Probes Supermix (Bio-Rad) to run quantitative PCR reactions as follows: 100 ng of cDNA mixed with 10 μL of Sso Advanced universal probes supermix(2x) and 1 μL of gene-specific hydrolysis probe in final volume of 20 μL. Reaction protocol for qPCR as follows: 30 seconds at 95 °C for cDNA denaturation, followed by 40 cycles of 15 seconds at 95 °C for denaturation, 30 seconds at 60 °C for annealing/extension. All cDNA synthesis and qPCR reactions were conducted using a CFX96 Real-Time System C1000 Touch Thermal Cycler (Bio-Rad). Control reactions without DNA template or reverse transcriptase did not show amplification. Technical replicates for all samples were performed in triplicates. Relative gene expression was measured using the ΔΔCT method.

12. Post-click labeling of G-SNAT by Cy5-azide for epifluorescence microscope imaging

For imaging with microscope, SB28 cells were seeded at about 50% confluence on cover glasses in a 6-well plate with complete medium a day before. After incubation at 37 °C for 3.5 h with 20 μM G-SNAT (from 10 mM stock in DMSO) and GD2-4-1BBζ CAR-T cells at a 1: 2 ratio in medium containing 1 % FBS, cells were washed 3 times with HBSS then fixed with 10% formalin for 30 min. Cells were permeabilized with PBS-Triton X100 (0.1% v/v) at room temperature for...
15 min then washed 3 times with PBS. Post-click assay solution was freshly prepared with 100 mM ascorbic acid (100 μL of 1 M stock in water, freshly prepared), 1 mM CuSO₄ (10 μL of 100 mM stock in water), 15 μM (BimC₄A)₃ (0.5 μL of 30 mM stock in water) and 5 μM Cy5-azide (1 μL of 5 mM in DMF) in 1 mL PBS. Cells were incubated with assay solution for 4 h at 37 °C then washed 3 times with PBS. Cells were then stained with 300 nM DAPI in PBS for 10 min at room temperature, washed with PBS 3 times and mounted on glass slides with antifade mounting medium.

The epifluorescence microscope images were acquired by 1X81 inverted microscope (Olympus) equipped with pE-4000 illumination systems (CoolLED) and ORCA-Flash4.0 digital CMOS camera (HAMAMATSU) with excitation at 405 nm for DAPI and 650 nm for Cy5. Digital images were reconstructed by MetaMorph software (v. 7.8.11.0) and analyzed using the ImageJ (NIH) software package.

13. Confocal microscope imaging

T cells were seeded in high density on poly-lysine treated cover glass in 6-well plate overnight. Cell suspension was gently removed before incubating with 5 μM G-SNAT-Cy5 in RPMI1640 medium for 2.5 h. After PBS washing, cells on cover glass were either stained with Hoechst and FITC conjugated CD3 antibody or fixed with PBS-Triton X100 (0.1% v/v) before stained with DAPI and rabbit anti-mouse granzyme B (ab4059, Abcam at 1:100 dilution in blocking buffer)/Alexa Fluor 488-conjugated goat anti-rabbit secondary (A11034, Life technologies at 1:200 dilution). Cover glass were washed and mounted with mounting medium for confocal imaging. Confocal images were obtained by a Zeiss LSM710 inverted confocal microscope, using a 20x, 40x/oil or 63x/oil immersion objective. All fluorescence images were gathered sequentially and stacked. Sequential Z sections of stained cells were recorded for generation of stacked images. Multi-channel 3D projections of fluorescent images were constructed from sequential Z sections of cells assembled in ImageJ.

14. Serum stability

G-SNAT-Cy5 (100 μM) was diluted in 100 μL mouse serum and incubated at 37 °C for 0, 30 min, 1, 2, 4, 6 and 8 h. Serum proteins were denatured by mixing cold methanol (900 μL) and precipitated by centrifugation at 8,000 g for 10 min. Supernatant were analyzed by HPLC (fluorescent detector, Ex640/Em670) and LC-MS. Percentage of tracer (relative area) was calculated as (peak area of tracer/total peak area on the HPLC chromatogram) x 100.

15. CAR T cell cytotoxic function study and cell viability assay

For cytotoxic function, 2.5 x 10⁵ A20Luc+ cells were incubated without or with effector CD19-28ζ CAR T cells at 1:10 and 1:1 effector-to-target (E: T) ratios in the presence of G-SNAT or G-SNAT-Cy5 at 2.5 μM, 5 μM or solvent control (DMSO, 1%) overnight in a 96-well black plate. The final volume was 200 μL. Next day, D-luciferin was added to 300 μg/mL final concentration before incubation at 37 °C for 5 min and scanned with an IVIS optical imager. The bioluminescence intensity was collected by defining the ROI on each well. Percent survival was calculated over the
bioluminescent intensity of A20Luc+ cells without CAR T cells in each treatment group.

Cell viability assay was performed with a CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, 2.5 x 10^5 A20Luc+, 2 x 10^5 activated untransduced CD8+ and 2 x 10^5 CAR T cells (70.7% transduction efficiency, so total 2.83 x 10^5 CAR T and CD8+ T cells) were incubated with G-SNAT (5 μM – 100 μM), G-SNAT-Cy5 (5 μM – 100 μM), and solvent (DMSO, 1%) in a 96 well-plate at 37 °C for 3.5 h in the presence of MTS reagent. The absorbance at 490 nm was collected on a plate reader and plotted.

16. In vivo imaging and data analysis

All experimental procedures using mice were performed in agreement with protocols approved by Institutional Animal Care and Use Committee (IACUC) of Stanford University as well as the USAMRMC Animal Care and Use Review (ACURO) in accordance with the laws of the United States and regulations of the Department of Agriculture. BALB/c and BALB/c Rag2–/–γc−/− mice were purchased from Jackson Laboratory and bred in house at the Research Animal Facility at Clark Center of the Stanford University (Stanford, CA).

For the A20 subcutaneous model, 1 x 10^6 A20 cells were injected subcutaneously into right upper flanks of 6-week-old female BALB/c Rag2–/–γc−/− mice in 90% Matrigel. Tumor burden was assessed by external caliper and calculated by use of the formula (Length x Width x height)/2. Tumors were grown to around 300 mm³ for imaging. 6 x 10^6 CD19-28ζ CAR T cells prepared from immunocompetent Fluc+ BALB/c mice were intratumorally injected in a volume of 200 μL in PBS. To adjust for the variable transduction efficiencies of CD19-28ζ retroviral vectors into CD8+ T cells, the concentrations of injected CD19-28ζ CAR T cells were normalized.

For the systemic lymphoma model, BALB/c mice were sublethally (4.4 Gy) irradiated. 1 x 10^6 A20 luc+/YFP cells were injected via tail vein. 2.4 x 10^6 CAR-T or CD8+T cells prepared from immunocompetent BALB/c mice were injected through the retro-orbital route. To adjust for the variable transduction efficiencies of CD19-28ζ retroviral vectors into CD8+ T cells, the concentrations of injected CD19-28ζ CAR T cells were normalized. Tumor burden was assessed by in vivo BLI^4. Briefly, 10 min after intraperitoneal injection of D-luciferin, mice were imaged for 5 min with an IVIS 100 charge-coupled device imaging system (Xenogen). For G-SNAT-Cy5, 5 nmol probes were injected through tail vein and imaged longitudinally with IVIS (Ex640/Em690) from 1 to 20 h. Images were analyzed with Living Image Software 2.5 (Xenogen). Intensity was collected by defining region of interest (ROI).

For the checkpoint blockade therapy model, 1 x 10^6 CT26 cells were injected subcutaneously into right upper flanks of 8-week-old female Fluc+ BALB/c mice in 50% Matrigel. Tumor burden was assessed by external caliper and calculated by use of the formula (Length x Width x height)/2. When reached ~200 mm³, tumors were treated with a combined regimen of anti-PD1 (200 μg) and anti-CTLA4 (100 μg) intraperitoneally for three rounds at day 9, 12, and 15. Treated mice with complete tumor regression were defined as responders. The mice were imaged sequentially with GBLI-2 (200 μg-i.v. retro orbital), G-SNAT-Cy5 (5 nmol-i.v. retro orbital, Ex), and D-luciferin (3 mg-i.p.), two hours apart, at day 9, 12, 15, 18, and 20 with a Largo-X imaging system. Images
were analyzed with Aura image software 3.2 (Spectral Instruments Imaging). Intensity was collected by defining ROI on tumors.

17. Biodistribution study

Healthy BALB/c (whole animal images) or C57Bl/6J (organ images) mice were injected with G-SNAT-Cy5 (retro orbital, 5 nmol) in PBS and imaged (Ex640/Em690) at 1 hour post injection with Largo X imaging system. After mice were euthanized, the organs were quickly excised and rinsed in sterile PBS and imaged. The fluorescence intensity of each organ was acquired by defining the ROI with Aura image software 3.2 (Spectral Instruments Imaging).

18. Immunofluorescent staining

Tissue preparation: The animals were anesthetized and sacrificed by a cervical dislocation after CO2 narcosis. Tumors were surgically removed post-mortem and fixed overnight in sterile 4% paraformaldehyde and transferred in 30% sucrose for 24 h. After removal of PFA by sucrose treatment, the tissues were cut to desired size and embedded in peel-a-way disposable base molds (ThermoFisher Scientific) containing O.C.T (Tissue-Tek O.C.T. Compound, Sakura Finetek) and frozen using dry ice. The embedded frozen tissues were cut to 10-micron thickness using CryotomeTM and the tissue slices were collected on Superfrost Ultra Plus glass slides (ThemoFisher Scientific). The glass slides that contained the tissue slices were stored at -20°C.

Histology: The tumor tissue slides were equilibrated to room temperature before any further manipulation. The slides were immersed in antigen retrieval solution containing 10 mM citric acid (monohydrate) and 0.05% Tween 20 at pH 6.0 and heated to 98 °C for 20 min. After elapse of 20 min, the slides were removed gently and cool-downed to room temperature, allowing reformation of antigenic sites after exposure to high temperature. The slides were washed twice using wash buffer (1x Tris Buffer Saline (TBS) plus 0.025% Triton X-100) with gentle shaking in orbital shaker. The tissues were then blocked using 10% goat serum in blocking buffer (1x TBS containing 1% BSA) for 2 h at room temperature, followed by overnight primary antibody treatment at 4 °C. The primary antibodies used were rabbit anti-mouse Granzyme B (ab4059, Abcam at 1:100 dilution in blocking buffer) and rat anti-mouse CD8a (MCA609GT, Bio-Rad at 1:100 dilution). After primary antibody staining, the tissues slides were washed twice and stained with secondary antibodies conjugated to fluorophore. The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-rabbit secondary (A11034, Life technologies at 1:200 dilution) and PerCP/Cy5.5-conjuagted goat anti-rat IgG secondary (405424, Biolegend at 1:100 dilution). This step was followed by twice washing and staining with primary antibody for CD19 pre-conjugated with fluorophore, Alexa Fluor 594-conjugated rat anti-mouse CD19 (115552, Biolegend at 1:100 dilution). The tissues slides were then stained with 300 nM DAPI solution for 20 min and air dried and mounted using ProLongTM Diamond anti-fade mountant (ThermoFisher Scientific) and imaged using a Zeiss LSM710 confocal microscope.

19. Statistical Analysis

GraphPad Prism 7 was utilized for plotting and statistical analysis. The significant difference was determined by performing t-test (Figure 4D) using unpaired nonparametric Mann-Whitney test,
one-way (Figure 4K, 4L, S12A), followed by Bonferroni’s multiple comparisons test, two-way
ANOVA followed by Tukey’s (Figure 4C, 5B, 5E, 5F, 5G) or Sidak (Figure S12B, S12C) multiple
comparisons test to determine the statistical significance with 95% confidence intervals with *p <
0.0332; **p < 0.0021, ***p < 0.0002, ****p < 0.0001, ns: not significant.
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Figure S1. Synthesis of G-SNAT precursor. Reagents and conditions: (a) 3-(Boc-amino)-1-propanol, Pd(OAc)$_2$, BINAP, Cs$_2$CO$_3$, reflux, 110 °C; (b) (i) 25% TFA in DCM, r.t., 30 min; (ii) 4-(Boc-aminomethyl)benzoic acid, HBTU, DIPEA, DMF, r.t., 2 h. (c) (i) 25% TFA in DCM, r.t., 30 min; (ii) Boc-propargyl-Gly-OH, HBTU, DIPEA, DMF, r.t., 2 h. (d) (i) 25% TFA in DCM, r.t., 30 min; (ii) Boc-Gly-OH, HBTU, DIPEA, DMF, r.t., 2 h. (e) (i) 25% TFA in DCM, r.t., 30 min; (ii) Boc Cys(SEt)-OH DCHA, HBTU, DIPEA, DMF, r.t., 2 h; (f) 25% TFA in DCM, r.t., 30 min.
Figure S2. Synthesis of G-SNAT and G-SNAT-Cy5. Reagents and conditions: (a) (i) HBTU, DIPEA, DMF, r.t., 2.5 h; (ii) CF3COOH: DCM: TIPS = 1: 1: 0.05, 5 mL, r.t., 2 h; (b) sulfo-Cy5-azide, CuSO4, sodium absorbate, (BimC4A)3, DMSO/HEPES = 1: 4.
Figure S3. Representative HPLC traces in grzmB and caspase-3 kinetic study. The enzymatic reaction kinetics and specificity studies by longitudinal monitoring of percentage conversion of G-SNAT (10 μM) into cleaved G-SNAT after incubation with equal amounts (100 U) of recombinant
Figure S4. Representative HPLC traces of 100 μM of G-SNAT-Cy5 incubated in mouse serum at different times (fluorescent detector Ex640/Em670). Samples were dissolved and precipitated in 80% methanol before HPLC analysis.
Fig. S5. Flow cytometry analysis of A20, untransduced CD8+ T, CAR T and Naïve T cells. Upper panel: Gating strategy used for identification of untransduced CD8+ and CAR T cells. Live cells were first identified and selected based on CD3+ (BV421) and CD8+ (APC/Cy7) staining. Final identification of CAR T cells was based on CAR (APC) staining. Representative histograms for the indicated cells, including a negative control with isotype staining is shown for untransduced CD8+ and CAR T cells. Lower left: Gating strategy used for identification of naïve T cells isolated from mice spleens. Live cells were first identified and selected based on CD3+ (BV421) and CD8+ (APC/Cy7) staining. Final identification of naïve T cells was based on CD44<sub>low</sub> (PE) and CD62<sub>high</sub> (APC) staining. Representative histograms for the indicated cells, including a negative control with isotype staining (Left) and staining with CD44 and CD62L. Lower right: The expression of CD19 on A20 cells was confirmed by staining with Alexa Fluor 594 conjugated CD19 antibody with naïve T cells (CD3+CD19-) as a control.
Figure S6. Microscopic imaging of CAR T cells incubated with G-SNAT-Cy5 (5 μM) for 2.5 h then fixed, permeabilized, and stained with rabbit anti-gzmB/Alexa Fluor 488-conjugated goat anti-rabbit secondary (green, Ex488/Em520) and DAPI (blue, Ex390/Em440). Magenta (Ex650/Em670) represents retained G-SNAT-Cy5.
Figure S7. Internalization of G-SNAT-Cy5 is dependent of endocytosis. G-SNAT-Cy5 (5 μM) were added to untransduced activated CD8+ T cells and incubated at 4 or 37 °C for 2.5 h before flow cytometry analysis. The MFI-Cy5 of viable cells were plotted in histogram.
Figure S8. GzmB activity under acidic condition. HPLC traces of G-SNAT-Cy5 incubated with gzmB (0.05 μg/ml) in MES buffer (pH5.5, blue) or Tris buffer (pH7.5, red) at 37 °C for 4 hours. 11.24% and 46.22% indicate the percentage conversion relative to G-SNAT-Cy5 peak obtained by calculating the percentage of peak area (mAU*min) of probe on the corresponding HPLC trace.
Figure S9. Bioluminescence assay confirmed the cytotoxic function of CD19-28ζ CAR T cells in the presence of G-SNAT or G-SNAT-Cy5. Bioluminescent intensity of target A20Luc+ cells cocultured without or with effector CD19-28ζ CAR T cells at 1:10 and 1:1 effector-to-target (E:T) ratios in the presence of G-SNAT, G-SNAT-Cy5 with indicated concentration or solvent (DMSO, 1%) overnight in 96-well plate. D-luciferin (300 μg/mL) was added before scan with an IVIS optical imager.
Figure S10. Cell viability study of G-SNAT and G-SNAT-Cy5. Viability assay (MTS) with G-SNAT, G-SNAT-Cy5 and solvent (DMSO, 1%) treated A2O\textsuperscript{Luc+}, CD8\textsuperscript{+} T and CAR T cells at 37 °C for 3.5 h. Absorbance at 490 nm was measured with MTS tetrazolium compound.
Figure S11. Gating strategy used for identification of A20, naïve CD8+ T, untransduced activated CD8+ T and CAR T cells.
Figure S12. Statistical analysis of the MFI-Cy5 acquired by repeated flow cytometry analysis. (A) A20 cells treated with or without naïve CD8+ T, activated untransduced CD8+ T and CAR T cells. (B) Naïve CD8+ T, activated untransduced CD8+ T and CAR T cells incubated with or without A20 cancer cells. 10,000 cells were analyzed. T cells were incubated with A20 cancer cells at a 1:1 ratio for 2.5 h. (C) G-SNAT-Cy5 treated A20, naïve CD8+ T, activated untransduced CD8+ T and CAR T cells with or without a gzmB inhibitor. Cells were preincubated with inhibitor for 2 h before G-SNAT-Cy5 incubation for additional 2.5 h. Error bars represent standard deviation. ***p < 0.0002, ****p < 0.0001, ns: not significant.
Figure S13. Illustration of the post-click labeling of gzm B activated G-SNAT (upper) and caspase-3 activated C-SNAT4 (lower) nanoaggregation by Cy5 azide.
Figure S14. Imaging gzmB and caspase 3 activity in GD2-4-1BBζ CAR T treated SB28-RFP/GD2 cells with G-SNAT-Cy5 and C-SNAT4-Cy5. GD2-4-1BBζ CAR-T cells were added to a single layer of SB28-RFP/GD2 cells at 2 to 1 ratio in the presence of G-SNAT (left, 20 μM) or C-SNAT4 (right, 20 μM) for 2.5 h. Cells were gently washed, permeabilized and processed for Cy5-azide post-click labeling. Cells were also stained with DAPI (blue, Ex405/Em460), fixed, and mounted for epifluorescence microscope imaging. Red (Ex650/Em670) represent cyclized and aggregated G-SNAT-Cy5. Scale bar indicates 50 μm.
Figure S15. Bioluminescence imaging (left panel) and quantification (right panel) with D-luciferin of A20 implanted, untransduced activated CD8+ T\textsuperscript{Luc+} or CD19-28ζ CAR T\textsuperscript{Luc+} cells treated tumor-bearing mice.
Figure S16. Longitudinal fluorescence imaging with G-SNAT-Cy5 (5 nmol, Ex650/Em670) of A20 implanted (bottom) and CD19-28ζ CAR T\textsuperscript{Luc+} cells (top) treated tumor-bearing mice at day 12.
Figure S17. Longitudinal bioluminescence imaging with D-luciferin of A20^{Fluc+} implanted (bottom), activated untransduced CD8\(^+\) T cells (middle) or CD19-28\(\zeta\) CAR T cells (top) treated tumor-bearing mice.
Figure S18. Illustration of the gzmB activated bioluminescent assay with GBLI2. FLuc-firefly luciferase.
Figure S19. Representative intensity scaled fluorescent images of mice organs showing distribution of G-SNAT-Cy5 at 1 h post i.v. injection in hearts, lungs, livers, kidneys, spleens, bones, brains, muscles, gallbladders, regenerative systems and urinary tracks, and gastrointestinal tracks. A PBS treated mouse was set as negative control (upper panel). The relative fluorescence intensity of each organ in G-SNAT-Cy5 treated mice were plotted, n = 3 (lower panel). Error bars represent standard deviation.
Figure S20. Longitudinal bright field imaging of nontreated (bottom), treated non-responder (middle) and responder (top) groups in Figure 5D. The yellow circles indicate tumors. Representative mice from each group were shown here.
Figure S21. Relative fluorescent or bioluminescent intensity of tumors imaged with G-SNAT-Cy5, D-luciferin or GBLI2 at day 9, 12, 15, 18, and 20 quantified by defining the ROIs on tumors.
Figure S22. Immunofluorescent staining analysis of the nontreated, treated responded and nonresponded tumors from Figure 5I. Scale bar indicates 50 μm.
