supplement

Targeting epigenetic regulation and post-translational modification with 5-Aza-2' deoxycytidine and SUMO E1 inhibition Augments T Cell Receptor Therapy

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Reagents table

Reagent or Resource	Source	Catalogue number	Dilution
Antibodies western blot			
SUMO1 mouse monoclonal	Cell Signaling Technology	21C7, Cat# 33-2400 RRID:AB_2198257	1/1000
SUMO2/3 mouse monoclonal	Universit of Iowa	8A2: RRID: AB_2198421	1/500
p-STAT Tyr701 rabbit monoclonal	Cell Signaling Technology	Cat# 9167; RRID: AB_561284	1/1000
β-actin mouse monoclonal	Sigma-Aldrich	Cat#: A5441 RRID: AB_476744	1/1000
Ubiquitin sc8017	Santa Cruz	Cat# sc-8017; RRID:AB_628423	1/5000
Antibodies Flowcytometry			
CD8 - FITC	BD biosciences	Cat# 555366 clone RPA-T8	1/40
Sytox - PacificBlue	BD biosciences	Lot 2369078	1/1000
Viability - ZombieRed	Biolegend	Cat# 423110 Lot B356980	1/1000
LAG-3 (CD223) - PerCP efluor 710	Invitrogen	Cat# 46-2239-42 Lot 2404262 Clone 3DS223H	1/80
hCD45 - V500	BD biosciences	Cat# 560777 Lot1088708 CLONE HI30	1/80
CD38 – BV605	BD biosciences	Cat# 740401 Lot 2101710 Clone HIT2	1/120
PD-1 (297) – BV786	BD biosciences	Cat# 563789 Lot 2060437 Clone EH12.1	1/100
HLA DR – Alexa700	BD biosciences	Cat# 560743 Lot 1313902 Clone F46-6	1/150
Ki67 – PECy7	BD biosciences	Cat# 561283 Lot 1211447 Clone B56	1/100
HLA A*02 – FITC	BD biosciences	Cat# 551285 Lot 1165046 Clone BB7.2	1/100
CD137 – APC	BD biosciences	Cat# 550890 Lot 1243782 Clone 4B4-1	1/75
mCD45 – PECy5	BD biosciences	Cat# 553082 Lot 1298554 Clone 30-F11	1/150
CD45 RA – PE Texas red	Invitrogen	Cat# MHCD45RA17 Lot 2206152 Clone MEM-56	1/200
CD8 – APC/H7	BD biosciences	Cat# 560179 lot2025113 clone SK1	1/250
CD25 – BV711	BD biosciences	Cat# 740776 Lot 2101714 Clone M-A251	1/100
PD-1L (CD274) – BV421	BD biosciences	Cat# 563738 Lot 9136926 Clone MIH1	1/40
ICOS – BV650	BD biosciences	Cat# 563832 Lot 2068020 Clone DX29	11/40
IRF-1 PE	BD biosciences	Cat# 566322 Lot 1270370 Clone 20/IRF-1	1/400

Brilliant stain buffer plus	BD biosciences	Cat# 566385 Lot 1307618	1/20
Compounds			
TAK981	Chemietek	CT-TAK981	
5-Aza-2'-deoxycytidine	Merck	A3656	
(2-Hydroxypropyl)-β-cyclodextrin (HPBCD)	Merck	H107	
qPCR primers	**Primer ordered**	**Sequence source if external**	
IFNγ FW GAGTGTGGAGACCATCAAGGAAG IFNγ Rev TGCTTTGCGTTGGACATTCAAGTC	Sigma	HP200586 OriGene	
IFN-β Fw GACATCCCTGAGGAGATTAAGCA IFN-β Rev CAACAATAGTCTCATTCCAGCCA	Sigma	Dr. Jochemsen (CCB, LUMC, NL)	
IFN-α Fw AGAAGGCTCCAGCCATCTCTGT IFN-α Rev TGCTGGTAGAGTTCGGTGCAGA	Sigma	HP214678 Origene	
TNF-α Fw CTCTTCTGCCTGCTGCACTTTG TNF-α Rev ATGGGCTACAGGCTTGTCACTC	Sigma	HP200561 Origene	
IFITM3 FW ATGTCGTCTGGTCCCTGTTC IFITM3 Rev GTCATGAGGATGCCCAGAAT	Sigma	[1]	
IRF7 FW TGGTCCTGGTGAAGCTGGAA IRF7 Rev GATGTCGTCATAGAGGCTGTTGG	Sigma	[1]	
STAT1-FW CAGCTTGACTCAAAATTCCTGGA STAT1-Rev TGAAGATTACGCTTGCTTTTCCT	Sigma	[1]	
IFIT-1 FW GCCTTGCTGAAGTGTGGAGGAA IFIT-1 Rev ATCCAGGCGATAGGCAGAGATC	Sigma	HP226398 Origene	
ISG15 FW CAGCGAACTCATCTTTGCCAGTA ISG15 Rev CCAGCATCTTCACCGTCAGG	Sigma	Dr. Jochemsen (CCB, LUMC, NL)	
ISG56 Fw GGGCAGACTGGCAGAAGC ISG56 Rev TATAGCGGAAGGGATTTGAAAGC	Sigma	Dr. Jochemsen (CCB, LUMC, NL)	
IFNAR1 FW TTGGTGCAAGAGGAAGAAGAA IFNAR1 Rev GTGACAGAGACCACCCATAAC	Sigma	[1]	
IL2 FW ACCAGGATGCTCACATTTAAGTTTT IL2 Rev TCCCTGGGTCTTAAGTGAAAGTTT	Sigma	[1]	
Perforin1 FW ACTCACAGGCAGCCAACTTTGC Perforin1 Rev CTCTTGAAGTCAGGGTGCAGCG	Sigma	HP227388 Origene	
GranzymeB FW CGACAGTACCATTGAGTTGTGCG GranzymeB Rev TTCGTCCATAGGAGACAATGCCC	Sigma	HP207492 Origene	
T-bet FW ATTGCCGTGACTGCCTACCAGA T-bet Rev	Sigma	HP210499 Origene	

GGAATTGACAGTTGGGTCCAGG			
IL-4 FW CCGTAACAGACATCTTTGCTGCC IL-4 Rev GAGTGTCCTTCTCATGGTGGCT	Sigma	HP200556 Origene	
IL-5 FW GGAATAGGCACACTGGAGAGTC IL-5 Rev CTCTCCGTCTTTCTTCTCCACAC	Sigma	HP200819 Origene	
IL-10 FW TCTCCGAGATGCCTTCAGCAGA IL-10 Rev TCAGACAAGGCTTGGCAACCCA	Sigma	HP200540 Origene	
SRPR FW CATTGCTTTTGCACGTAACCAA SRPR Rev ATTGTCTTGCATGCGGCC	Sigma	Dr. Jochemsen (CCB, LUMC, NL)	
SDHA FW GCATTTGGCCTTTCTGAGGC SDHA Rev CTCCATGTTCCCCAGAGCAG	Sigma	[2]	
18s-RNA FW GGAGTATGGTTGCAAAGCTGA 18s-RNA Rev ATCTGTCAATCCTGTCCGTGT	Sigma	Dr. Jochemsen (CCB, LUMC, NL)	

Materials and Methods

Compounds

5-Aza-2'-deoxycytidine (5-Aza-2', Merck) was dissolved in DMSO for in vitro usage and in 20% (2-Hydroxypropyl)-β-cyclodextrin (HPBCD, Merck) for in vivo purposes. TAK981 (Chemietek) was dissolved in DMSO for *in vitro* usage and in 20% HPBCD for in vivo purposes. 5-Aza-2'-deoxycytidine (5-Aza-2', Merck) was dissolved in DMSO for in vitro usage and in 20% HPBCD for in vivo purposes.

Cell culture

OCI-AML3 cells (HLA-A*02:01+ mutant NPM1+ HA2+) were obtained from DSMZ (Braunschweig, Germany) and U266 cell line (HLA-A*02:01+, HLA-B*07:02+, BOB1+, MAGE-A1+) was obtained from Prof. Dr T. Mutis (Department of Hematology, VUMC, NL). Cell lines were cultured in IMDM (Lonza), supplemented with 1.5% glutamine (Lonza), 10% fetal bovine serum (Gibco, Life Technologies) and 1% Penicillin-Streptomycin (Lonza). Cells were cultured at 37 ˚C and 5% CO_2 in a humidified incubator.

T cells were cultured in IMDM (Lonza) supplemented with 5% fetal bovine serum (FBS; Gibco, Life Technologies), 5% human serum, 1.5% glutamine (Lonza), 1% penicillin/streptomycin (Lonza), and

100 IU/ml IL-2 (Proleukin; Novartis Pharma), herein referred to as T cell medium (TCM). CD8+ T cells were isolated from healthy donor peripheral blood mononuclear cells (PBMCs) by MACS using anti-CD8 MicroBeads (Miltenyi Biotec). CD8+ T cells were subsequently activated with irradiated autologous PBMCs (35 Gy) and 0.8 mg/mL phytohemagglutinin (PHA; Oxoid Microbiology Products, Thermo Fisher Scientific), and cultured in TCM. PBMCs were obtained from the Leiden University Medical Center Biobank for Hematological Diseases (approval number B16.039). Samples were collected after written informed consent.

TCR transfer to healthy donor T cells

On day 2 post activation, CD8+ T cells were retrovirally transduced with NPM1-TCR$^{(CLA\ A2)3}$, HA2-TCR$^{(YIG\ A2)4}$, BOB1/4G11-TCR$^{(APA\ B7)5}$, MAGE-A1-TCR$^{(KVL\ A2)6}$ or CMV-TCR$^{(NLV\ A2)3}$, using 24-well non-tissue culture plate coated with retronectin (30 mg/mL) (Takara) overnight at 4°C. Wells were blocked with 2% human serum albumin (HSA) (Sanquin) for 30 min. Viral supernatant was thawed and added to the 24-wells plate and spun for 20 min, 2,000g at 4˚C. Virus supernatant was removed and 0.3*10^6 activated CD8+ T cells were transferred to each well. After overnight incubation, T cells were transferred to a tissue culture plate. On day 7 post activation TCR transduced T cells were indirectly MACS enriched for introduced TCR expression on anti-mouse TCR-Cβ APC antibody (mTCR APC; BD Pharmingen) followed by anti-APC MicroBeads (Milenyi Biotec). Purified T cells were used in experiments between day 10-14 after activation. TCR expression was assessed by HLA tetramer binding; cells were stained for anti-mTCR APC antibody and PE labeled pHLA-tetramers. Cells were measured on the LSR II (BD Bioscience), and data were analyzed with FlowJo Version 10 software.

Viability assay

OCI-AML3 and U266 cells were seeded in 96-well flat bottom plate format in a density of 1*10^5 cells/mL. OCI-AML3 cells were treated for 4 days with increasing concentrations of TAK981 (0.0001 – 0.1 μM) or 5-Aza-2’ (0.025 – 20 μM) as indicated in the figures; 0.01% DMSO was used as control. For

synergy analysis, a dose range of 5-Aza-2' (0.025 – 0.10 µM) with or without 0.01 µM of TAK981 was used. For synergy response of U266, cells were treated with a dose range of 5-Aza-2' (1.5 – 20 µM) with or without 0.25 µM of TAK981. Presto Blue viability reagent (A13261, Merck) was added 1:10 into cell culture medium for 1 hour at 37°C and 5% CO_2. Fluorescence was measured with a plate reader (Victor X3, Perkin Elmer) at 544/591nm. Three technical replicates were used within each of three biological replicates performed for the viability assays performed. The excess overbliss model [7] was used to calculate the synergistic score, using the following formula with Fa as the fractional activity: Excess overbliss = (Fa1+2 − [(Fa1 + Fa2) − (Fa1 × Fa2)]) × 100.

Western Blot

Total cell lysates of OCI-AML3 and U266 cells treated with TAK981 (0.05 to 1 µM) or DMSO 0.01% were analyzed by western blotting for SUMO2/3, SUMO1 and conjugation. CD8+ T cells treated with 100 nM TAK981 and/or 250 nM 5-Aza-2' overnight were analyzed by western blotting for p-STAT Tyr701 and SUMO2/3 and β-actin for loading control. Total lysates were prepared on ice in SNTBS buffer (2% SDS, 1% NP40, 50mM Tris pH 7.5, 150 mM NaCl) followed by 10 min at 100˚C. Proteins were size separated with precast 4-12% Bis-Tris gradient gels (Thermo Fisher Scientific). Size-separated proteins were transferred to nitrocellulose membranes (0.45 µm, Amersham Protran Premium (Merck)). Membranes were incubated with primary antibodies against SUMO2/3 (1:500, mouse monoclonal 8A2, University of Iowa), SUMO1 (1:1000, 4930P, Cell Signaling Technology), ubiquitin (1:5000, sc8017, Santa Cruz), and β-actin in 5% milk powder in PBS - 0.05% Tween20. Membranes were incubated with p-STAT1 Tyr701 (1:1000, 58D6, Cell Signaling Technology) antibody in TBS – 0.05% Tween20 – 3% bovine serum albumin. Goat anti- mouse IgG- HRP (1:2500) and Donkey anti- rabbit IgG- HRP (1:10 000) were used as secondary antibodies in 5% milk in TBS – 0.05% Tween20 – 3% bovine serum albumin. ECL signal was detected using Pierce ECL2 (Life Technologies) and imaged using the iBright CL1500 (Invitrogen iBright Imaging Systems).

qPCR

CD8+ T cells from three healthy donors were cultured in TCM and treated with 10 and 100 nM TAK981 and/or 25 and 250 nM 5-Aza-2' or control DMSO 0.01% overnight, 10 days post stimulation. Total RNA of CD8+ T cells was isolated with use of SV total RNA isolation system (Promega). 0.5 – 1 µg of RNA was used for cDNA synthesis using random primers (Invitrogen) and reverse transcriptase ImProm-II (Promega) following manufacturer's protocol. Real-time quantitative PCR was performed with SYBR Green PCR Mastermix (Applied Biosystems) on a CFX384 real-time PCR detection system (Bio-Rad) according to the following protocol. 95 °C for 7 minutes, followed by 39 cycles of; 95 °C 15 seconds, 60 °C 15 seconds, 60 °C 35 seconds + measurement, the protocol was finalized with 95 °C 10 seconds, 65 °C 5 seconds + measurement and 95 °C 50 seconds. CT values of genes were normalized against the geometric mean of housekeeping genes (SRPR, 18S-RNA and SDHA). Primer sequences are listed in the reagent table.

Co-culture IFNγ ELISA

5000 CD8+ T cells (NPM1-TCR, CMV-TCR) were co-cultured with OCI-AML3 as target cells in an E:T ratio of 1:6. CD8+ T cells or OCI-AML3 target cells were pre-treated with 10 nM TAK981 and/or 250 nM 5-Aza-2' or 0.01% DMSO control, on day 10 and 14 post stimulation of CD8+ T cells. Subsequently OCI-AML3 and CD8+ T cells were washed and co-cultured overnight in 60 uL of TCM. Supernatant was harvested, diluted 5x and 125x and, IFNγ was measured by ELISA according to manufacturer's instructions adapted for 384 well plates (Diaclone).

CD8+ survival assay

Activated CD8+ T cells (NPM1-TCR, CMV-TCR, HA2.5-TCR) were co-cultured with irradiated (50Gy) target cells (OCI-AML3, OCI-AML2) for 5 and 7 days in 96-well round bottom plates in an E:T ratio of 1:5 (5,000 CD8+ T cells). During co-culture, cells were treated on day 1 and 4 with 10 nM TAK981 and/or 250 nM 5-Aza-2' or DMSO 0.01% as control. CD8+ T cell counts were measured with help of

flow cytometry (LSR-II). Cells were spun down in plates and CD8+-FITC conjugated ab (BD Pharmingen) was added for 30 min. Subsequently, cells were washed with PBS and resuspended in SytoxBlue (Thermofisher) dead marker (1:1000). Target cells have an internal label of tdTomato, which was used for identification. Each sample was run for a standardized time of 23 seconds. CD8+ T cells were gated out as presented in supplementary figure 3 and total counts were used for analysis.

***in vivo* tumor therapy**

In vivo studies performed were approved by the national Ethical Committee for Animal Research (AVD116002017891). OCI-AML3-Luc and U266-Luc cells were transduced with Luciferase-tdTomato. Cell lines were bulk enriched for tdTomato expression using an Aria III cell sorter (BD Biosciences) to reach >98% purity. Male and female NOD *scid* gamma (NSG) mice (NOD.Cg-Prkdc(scid) Il2rg(tm1Wjl)/SzJ) originated from the Jackson Laboratory and were bred in house. Male NSG mice were inoculated with 1*10^6 OCI-AML3-Luc cells intravenously (i.v.). Male and female NSG mice were inoculated with 2*10^6 U266-Luc (multiple myeloma) cells i.v.

Tumor growth was measured bi-weekly with use of the In Vivo Imaging System (IVIS-spectrum, Perkin Elmer). Mice were subcutaneously injected with 150 µL of 7.5 mM D-luciferin potassium salt (Synchem) and bioluminescence (photons/sec/cm2/r) of U266-LUC and OCI-AML3 cells was measured.

Treatment with TAK981 (25mg/kg) and/or 5-Aza-2' (2.5mg/kg) or HPBCD-buffer as control was started on day 10 or day 14 post inoculation of OCI-AML3 and U266 respectively. Maximum weight of mice used for drug doses calculation was set to 20gram. For the OCI-AML3 model, 3*10^6 NPM1-TCR, HA2-TCR or CMV-TCR T cells were injected i.v. on day 15. For the U266 model, 1*10^6 BOB1-TCR, MAGE-A1-TCR or CMV-TCR T cells were injected i.v. on day 19. Drug treatment was continued bi-weekly until day 50 post tumor inoculation.

In vivo NPM1-TCR CD8+ T cell-LUC tracking

Male NSG-mice (n=6/group) were inoculated with 1*10^6 OCI-AML3 cells via i.v. injection. Treatment with TAK981 (25mg/kg) and/or 5-Aza-2' (2.5mg/kg) or HPBCD-buffer control was started on day 14 post inoculation of OCI-AML3 cells and continued bi-weekly. Maximum weight of mice used for drug doses calculation was set to 20gram. NPM1-TCR and CMV-TCR CD8+ T cells were transduced with Luciferase-tdTomato. CD8+ T cells were bulk enriched for tdTomato expression using an Aria III cell sorter (BD Biosciences) to reach >98% purity. On day 18 post OCI-AML3 injection 3*10^6 NPM1-TCR luc or CMV-TCR Luc CD8+ T cells were injected i.v.. T cell BLI was monitored on day 3, 6 and 9 post injection, using IVIS imaging following s.c. injection of 150 µL of 7.5 mM D-luciferin.

Isolation of bone marrow and ex-vivo CD8+ T cell and tumor analysis

Bone marrow was harvested from euthanized mice on day 2 (n=4/group), day 5 (n=3/group) and day 8 (n=3/group). Femurs were cleaned of surrounding tissue and cut open on the knee-side. The open femurs were placed into a 1.5ml Eppendorf tube containing 100ul of T cell medium and spun down at 2500g at room temperature. Bone marrow suspension was filtered through a cell strainer (70µM) (sysmex) into a sterile tube. Subsequently, bone marrow was spun down (500g) and resuspended in 500 µL red blood cell lysis buffer (154 mM ammoniumchloride) for 10 minutes on ice. Lysed samples were spun and supernatant was removed. Samples were transferred to round bottom 96-well plates for staining. 20 µL of Zombie-red staining (Thermofisher) (1:1000 in PBS) was added to each well for 25 min. Plates were washed with PBS and 100 µL of paraformaldehyde (1%) was added and incubated for 8 min at room temperature for fixation. Plates were spun and PFA was removed from samples; subsequently 100 µL saponin-buffer (500 mL PBS, 2 mL 200g/L albumin, 1% P/S, 0.1% saponin (Quillaja)) was added and plates were incubated for 30 min at 4˚C. After removal of the saponin-buffer, 20 µL of antibody mix (reagent table) including BV staining buffer Buffer (BD Pharmingen) plus (1/20) and 5% normal mouse serum (Thermofisher) was added and incubated for 30 min at room temperature. Finally, plates were washed with saponin-buffer and the samples were resuspended in

50 µL saponin-buffer and aquired on a Cytek Aurora spectral flow cytometer 3L (Cytekbio). Bone marrow of mice with only tumor was used as unstained samples.

Isolation of bone marrow and ex-vivo single cell tumor analysis

Bone marrow was harvested as stated above. For each mouse bone marrow of both paws was combined. Negative MACS enrichment of human cells of the bone marrow was performed, using anti-mCD45 (APC) and APC-beads. Enriched human cells from each mouse were labeled with unique hashtags (totalSeq-A human hashtags). Subsequently, to further purify the sample, anti-hCD45 was used to positive select human cells through sorting. Following 1 run of 10x Genomics 3' v3.1 chemistry was performed.

Single cell RNA sequencing

scRNA-seq was performed on sorted human cells. Single-cell gene expression libraries were prepared using the 10x Genomics Chromium X platform, specifically employing the Chromium Next GEM Single Cell 3' Library & Gel Bead Kit v3.1 and Chromium Next GEM Chip G Single Cell Kit (10x Genomics) according to manufacturer's instructions. Subsequently, the gene expression libraries were sequenced on a NovaSeq 6000 S4 flow cell with v1.5 chemistry (Illumina), and fastq files were generated using Cell Ranger mkfastq (10x Genomics). These fastq files were analyzed using the 10x Genomics Cell Ranger software version 7.0.0 and a custom reference with GRCh38-2020 containing the tdTomato gene. Raw data will be made available on GEO.

All downstream analysis was performed using Seurat (v.4.3.0). Samples were demultiplexed using hashtag oligos (HTO) by application of the 'MULTIseqDemux' function to identify singlets, doublets and negative cells [8]. Cells with a low number of expressed genes (<200) or high mitochondrial content (> 10%) were filtered out from the analysis. Data from a total of 2075 remaining cells was normalized using Seurat's 'LogNormalize' function with scaling factor set at 10,000. Variable features were identified using the 'FindVariableFeatures' function, resulting in the selection of 2,000 genes. To

visualize cells in a two-dimensional space, t-distributed stochastic neighbor embedding (t-SNE) was used. Differential gene expression was performed using the 'FindAllMarkers' function, with a min.pct of 0.25 and logfc. threshold at 0.25. We identified a cluster of 1795 tumor cells and a separate cluster of T cells based on expression of T cell genes and expression of tdTomato. T cell counts were insufficient for proper analysis and therefor excluded from the results. Data from all tumor cells were extracted and analyzed separately. Selected genes of interest associated with proliferation, MHC, cell surface molecules, and immune checkpoints were visualized per treatment condition using the 'DotPlot' function.

Supplementary References

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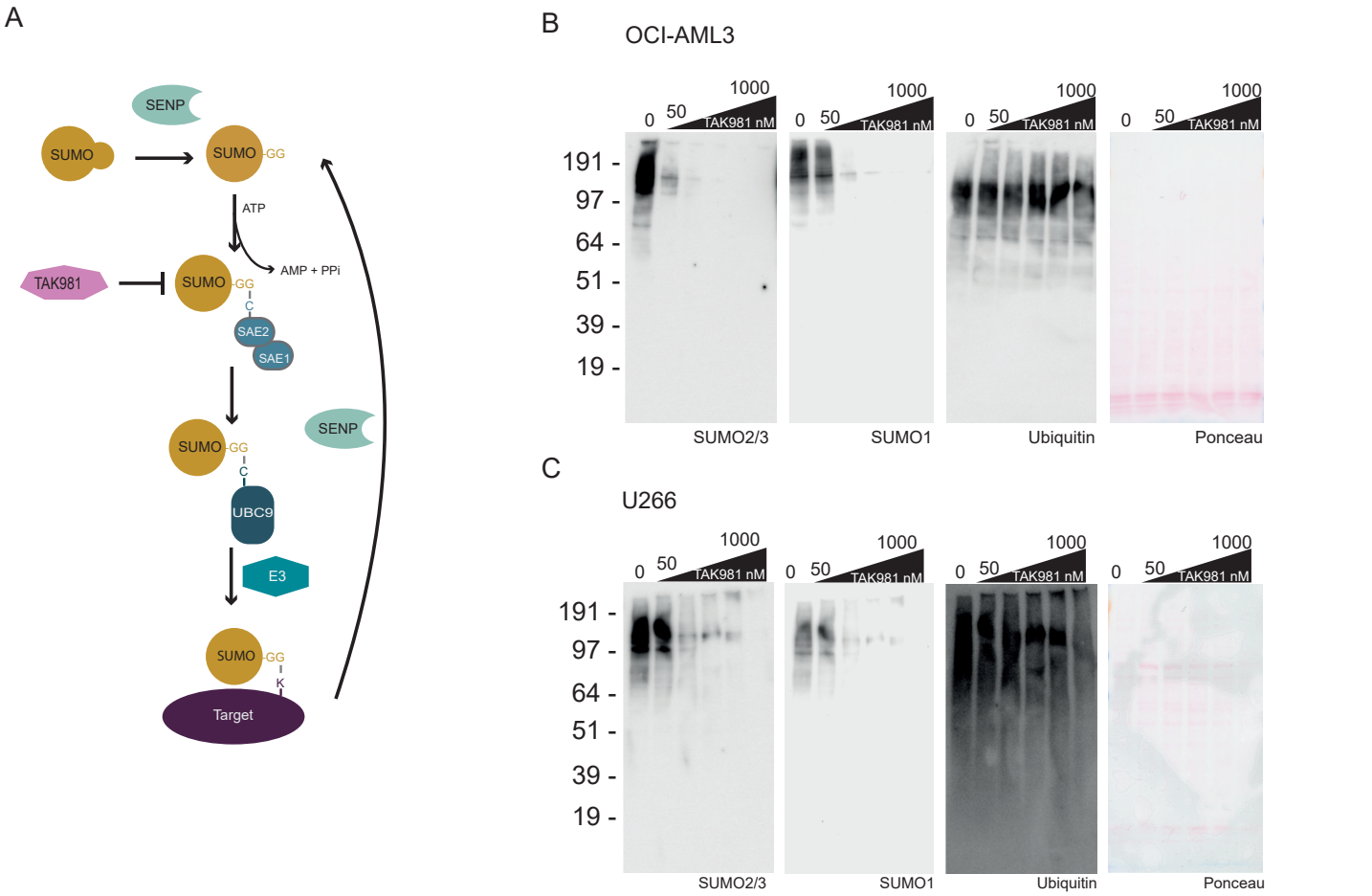

A
SENP
SUMO
SUMO -GG
ATP
AMP + PPi
TAK981
SUMO -GG
C
SAE2
SAE1
SUMO -GG
C
SENP
UBC9
E3
SUMO -GG
K
Target
B
OCI-AML3
0 50 1000 TAK981 nM
191 -
97 -
64 -
51 -
39 -
19 -
SUMO2/3
SUMO1
Ubiquitin
Ponceau
C
U266
SUMO2/3
SUMO1
Ubiquitin
Ponceau
D
U266
Viability ratio to control
5-Aza-2' [µM]
E
U266
Viability ratio to control
TAK981 [uM]
F
0nM TAK981
0.025 µM TAK981
Excess overbliss 0.025 µM TAK981
U266
Viability ratio to control
Excess overbliss (%)
5-Aza-2' [µM]

13

Figure S1

Supplementary Figure 1 TAK981 and 5-Aza-2' synergistically reduce U266 viability. **A** The SUMOylation cycle. SUMO precursor protein is cleaved by SUMO specific proteases (SENPs) to produce mature SUMO. Target proteins are SUMOylated via an enzymatic cascade that consists of the E1 activating enzyme, the E2 conjugating enzyme and an E3 ligase. SUMO can be removed from a target protein by SENPs. Small molecule SUMOylation inhibitor TAK981 inhibits the E1 enzyme. B OCI-AML3 cells treated with TAK981 (50 – 1000 nM) or DMSO 0.01% control for 4 hours. Cells were lysed and analysed by immunoblotting for SUMO2/3, SUMO1 and ubiquitin. PonceauS staining was used as loading control. **C** U266 cells treated with 50 – 1000 nM of TAK981 or DMSO 0.01% control for 4 hours, were analysed for SUMO2/3, SUMO1 and ubiquitin protein expression via Western Blotting. PonceauS staining was used as loading control. **D** U266 cell viability is shown after 4 days of 5-Aza-2' treatment (0.025 – 20 μM) or control DMSO 0.01% treatment (n=3). **E** U266 cell viability after 4 days of TAK981 treatment (0.05 – 1 μM) or control DMSO 0.01% treatment. (n=3). **F** U266 cell viability after 4 days of combination treatment with dose response range of 5-Aza-2' with 25 nM of TAK981. Excess overbliss synergy calculations of single 5-Aza-2' doses versus 5-Aza-2' doses with 25 nM TAK981 are shown in the right y-axis per dose.

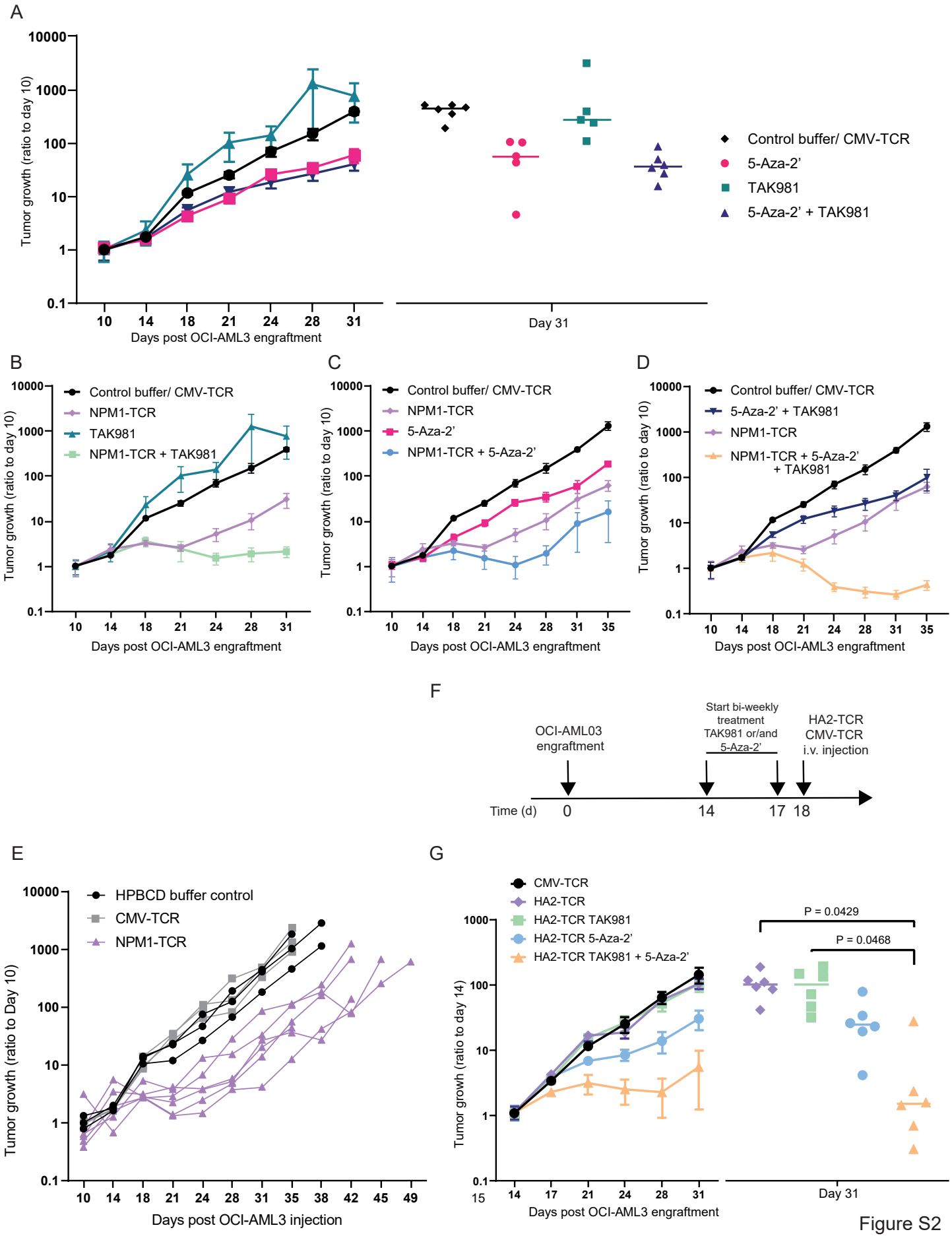

A
Tumor growth (ratio to day 10)
Days post OCI-AML3 engraftment
Day 31
Control buffer/ CMV-TCR
5-Aza-2'
TAK981
5-Aza-2' + TAK981
B
Control buffer/ CMV-TCR
NPM1-TCR
TAK981
NPM1-TCR + TAK981
C
Control buffer/ CMV-TCR
NPM1-TCR
5-Aza-2'
NPM1-TCR + 5-Aza-2'
D
Control buffer/ CMV-TCR
5-Aza-2' + TAK981
NPM1-TCR
NPM1-TCR + 5-Aza-2' + TAK981
F
OCI-AML03 engraftment
Start bi-weekly treatment TAK981 or/and 5-Aza-2'
HA2-TCR CMV-TCR i.v. injection
Time (d)
0
14
17
18
E
HPBCD buffer control
CMV-TCR
NPM1-TCR
Tumor growth (ratio to Day 10)
Days post OCI-AML3 injection
G
CMV-TCR
HA2-TCR
HA2-TCR TAK981
HA2-TCR 5-Aza-2'
HA2-TCR TAK981 + 5-Aza-2'
Tumor growh (ratio to day 14)
Days post OCI-AML3 engraftment
P = 0.0429
P = 0.0468
Day 31

Figure S2

Supplementary Figure 2 NPM1-TCR CD8+ T cells and HA2-TCR CD8+ T cells anti-tumor efficacy is enhanced by 5-Aza-2' and TAK981 *in vivo*. **A** OCI-AML3 tumor outgrowth average per group (n=6); ratio to bioluminescent (BLI photons/sec/cm2/r) signal per mouse at day 10 is shown. Graphs represent time point when all mice were present in the experiment. Only compound groups from Figure 3B are shown. One-Way ANOVA analysis was performed for tumor signals at day 31, in GraphPad Prism 9.3.1. **B** TAK981 enhances tumor cell killing by NPM1-TCR. Data from Figure 3B. OCI-AML3 tumor outgrowth average per group (n=6) ratio to bioluminescent (BLI photons/sec/cm2/r) signal per mouse at day 10. Graphs represent time point when all mice were present in the experiment. **C** 5-Aza-2' enhances tumor cell killing by NPM1-TCR. Data from Figure 3B. OCI-AML3 tumor outgrowth average per group (n=6); ratio to bioluminescent (BLI photons/sec/cm2/r) signal per mouse at day 10. Graphs represent time point when all mice were present in the experiment. **D** TAK981 and 5-Aza-2' enhance tumor cell killing by NPM1-TCR. Data from Figure 3B. OCI-AML3 tumor outgrowth average per group (n=6) ratio to bioluminescent (BLI photons/sec/cm2/r) signal per mouse at day 10. Graphs represent time point when all mice were present in the experiment. **E** Control groups of Figure 2B & C, Supplementary Figure 2 A-D plotted separately to indicate the non-responsiveness of the control buffer and CMV-TCR to OCI-AML3 tumor compared to NPM1-CMV-TCR. **F** Timeline of *in vivo* experiment shown in Figure 3G. Luciferase expressing OCI-AML3 cells (1*10^6) were injected intra-venously (i.v.) into the tail vain of NSG-mice and engrafted for 14 days. Drug dosing was used as described in Figure 3A. HA2-TCR CD8+ T cells (3*10^6) were injected on day 18. **G** OCI-AML3 tumor outgrowth average per group (n=6); ratio to bioluminescent (BLI photons/sec/cm2/r) signal per mouse at day 10. Graphs represent time point when all mice were present in the experiment. One-Way ANOVA analysis was performed for tumor signals at day 31, in GraphPad Prism 9.3.1.

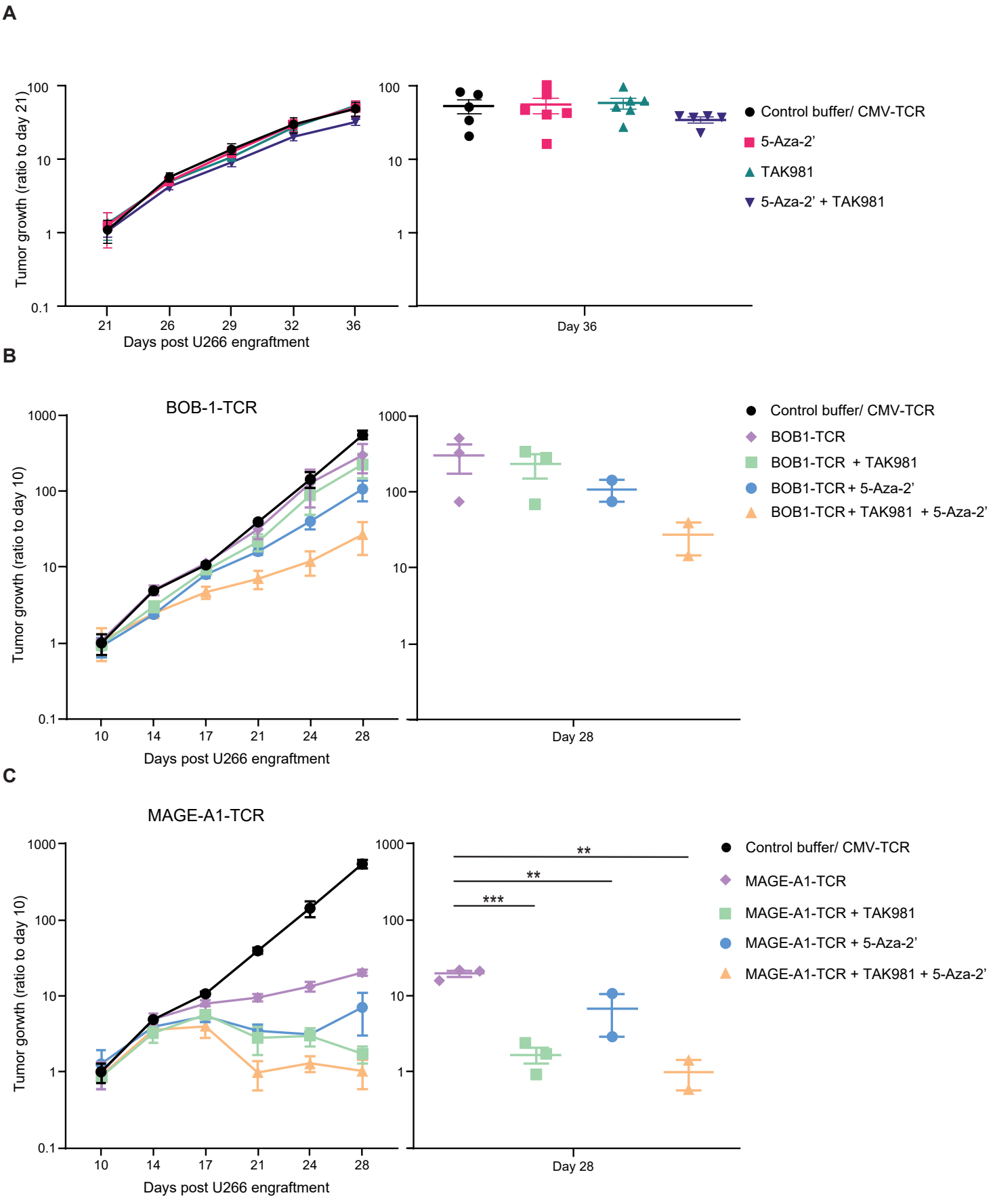
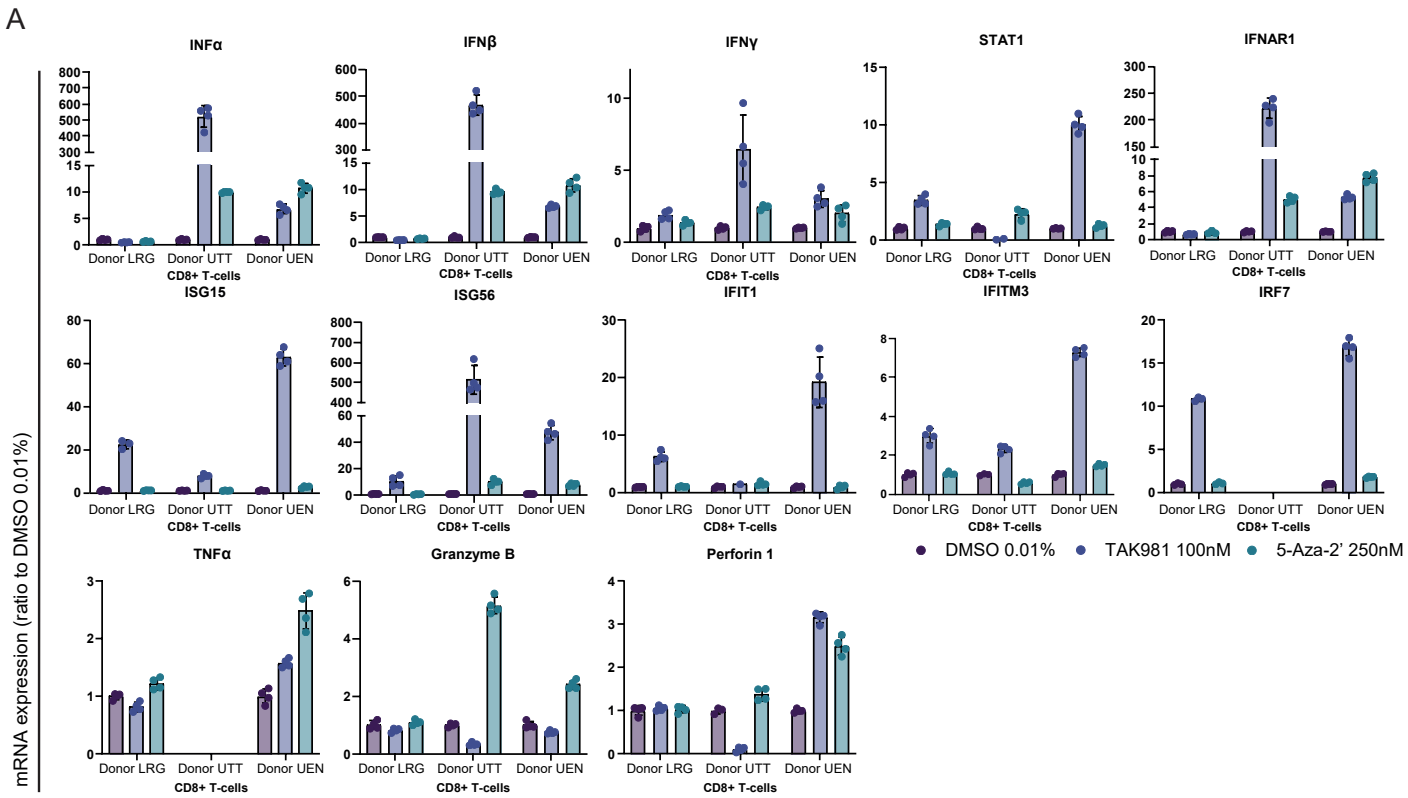
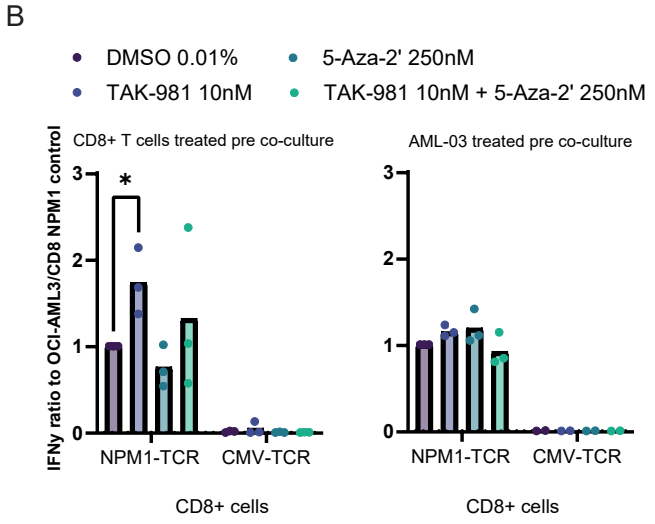
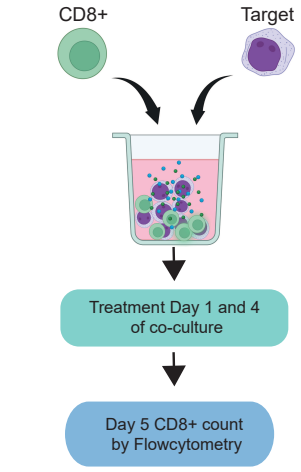

A
Tumor growth (ratio to day 21)
100
10
1
0.1
21
26
29
32
36
Days post U266 engraftment
Day 36
Control buffer/ CMV-TCR
5-Aza-2'
TAK981
5-Aza-2' + TAK981
B
BOB-1-TCR
Tumor growth (ratio to day 10)
1000
100
10
1
0.1
10
14
17
21
24
28
Days post U266 engraftment
Day 28
Control buffer/ CMV-TCR
BOB1-TCR
BOB1-TCR + TAK981
BOB1-TCR + 5-Aza-2'
BOB1-TCR + TAK981 + 5-Aza-2'
C
MAGE-A1-TCR
Tumor gorwth (ratio to day 10)
**
**
***
Day 28
Control buffer/ CMV-TCR
MAGE-A1-TCR
MAGE-A1-TCR + TAK981
MAGE-A1-TCR + 5-Aza-2'
MAGE-A1-TCR + TAK981 + 5-Aza-2'

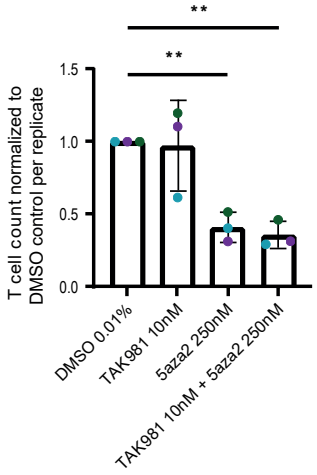
Figure S3

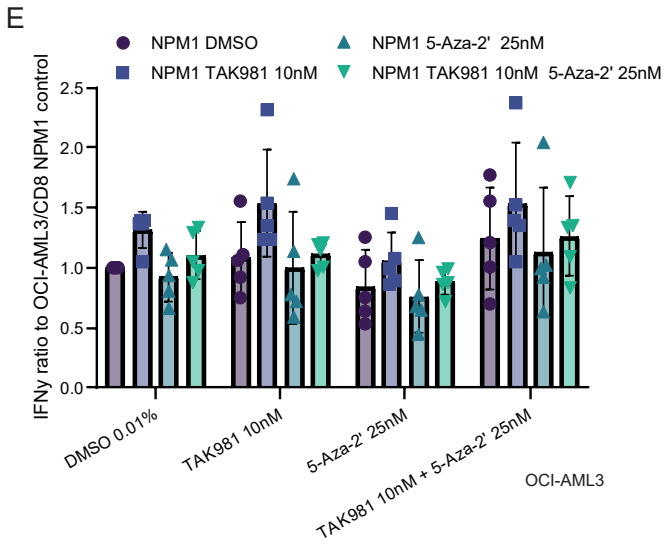
Supplementary Figure 3 MAGE-A1 and BOB1-TCR CD8+ T cell anti-tumor efficacy in a multiple myeloma model (U266) is enhanced by 5-Aza-2' and TAK981 *in vivo*. Luciferase expressing U266 cells (2*10^6) expressing BOB1 and MAGE-A1 and positive for HLA-B*07:02 and HLA-A*02:01 were injected intra-venously (i.v.) into the tail vain of NSG-mice and engrafted for 14 days. Tumor volume was measured by IVIS. At day 14 treatment was started. Two rounds of the drug treatment with TAK981 (25 mg/kg) and/or 5-Aza-2' (2.5 mg/kg) were carried out. Subsequently 4G11/BOB1-TCR or MAGEA1-TCR CD8+ T cells (1*10^6) were i.v. injected on day 19. **A** U266-Luc tumor outgrowth per group (n=6) NSG (male) mice. Data is presented as ratio to bioluminescent (BLI photons/sec/cm2/r) signal per mouse at day 21. Graphs represent time point when all mice were present in the experiment. Only groups treated with compound are shown. One-Way ANOVA analysis was performed for tumor signals at day 36, in GraphPad Prism 9.3.1. **B** U266-Luc tumor outgrowth average per group (n=3) NSG (female) mice data is presented as ratio to bioluminescent (BLI photons/sec/cm2/r) signal per mouse at day 10 upon BOB1-TCR with or without TAK981 and/or 5-Aza-2'. One-Way ANOVA analysis was performed for tumor signals at day 28, in GraphPad Prism 9.3.1. **C** U266-Luc tumor outgrowth average per group (n=3) NSG (female) mice data is presented as ratio to bioluminescent (BLI photons/sec/cm2/r) signal per mouse at day 10 upon MAGE1-A2-TCR with or without TAK981 and/or 5-Aza-2'. One-Way ANOVA analysis was performed for tumor signals at day 28, in GraphPad Prism 9.3.1.


A
INFα
IFNβ
IFNγ
STAT1
IFNAR1
ISG15
ISG56
IFIT1
IFITM3
IRF7
TNFα
Granzyme B
Perforin 1
Donor LRG
Donor UTT
Donor UEN
CD8+ T-cells
mRNA expression (ratio to DMSO 0.01%)
DMSO 0.01%
TAK981 100nM
5-Aza-2' 250nM


B
DMSO 0.01%
5-Aza-2' 250nM
TAK-981 10nM
TAK-981 10nM + 5-Aza-2' 250nM
CD8+ T cells treated pre co-culture
AML-03 treated pre co-culture
IFNγ ratio to OCI-AML3/CD8 NPM1 control
NPM1-TCR
CMV-TCR
CD8+ cells


C
CD8+
Target
Treatment Day 1 and 4 of co-culture
Day 5 CD8+ count by Flowcytometry


D
T cell count normalized to DMSO control per replicate
DMSO 0.01%
TAK981 10nM
5aza2 250nM
TAK981 10nM + 5aza2 250nM


E
NPM1 DMSO
NPM1 5-Aza-2' 25nM
NPM1 TAK981 10nM
NPM1 TAK981 10nM 5-Aza-2' 25nM
IFNγ ratio to OCI-AML3/CD8 NPM1 control
DMSO 0.01%
TAK981 10nM
5-Aza-2' 25nM
TAK981 10nM + 5-Aza-2' 25nM
OCI-AML3

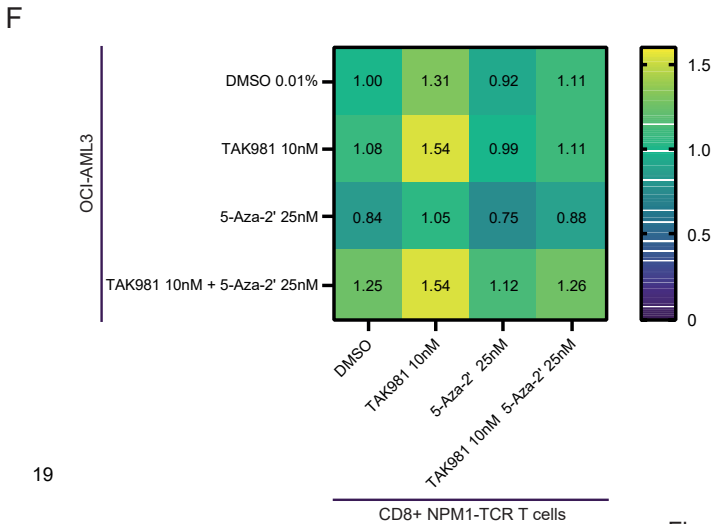

F
OCI-AML3
DMSO 0.01%
TAK981 10nM
5-Aza-2' 25nM
TAK981 10nM + 5-Aza-2' 25nM
1.00 1.31 0.92 1.11
1.08 1.54 0.99 1.11
0.84 1.05 0.75 0.88
1.25 1.54 1.12 1.26
DMSO
TAK981 10nM
5-Aza-2' 25nM
TAK981 10nM 5-Aza-2' 25nM
CD8+ NPM1-TCR T cells

Figure S4

Supplementary Figure 4 Higher dosing of TAK981 and 5-Aza-2' increasingly activate interferon signalling, also causing more cytotoxicity. **A** mRNA expression levels of IFNα, IFNβ, IFNγ, STAT1, IFNAR1, ISG15, ISG56, IFIT1, IFITM3, IRF7, TNFα, Granzyme B and Perforin 1 were measured using qPCR, for CD8+ T cells isolated from three different healthy donors. CD8+ T cells were treated 10 days post stimulation with 100 nM TAK981, 250 nM 5-Aza-2' or DMSO 0.01% as control overnight. 18sRNA, SDHA and SRPR were used as housekeeping genes. Expression was plotted as ratio to DMSO 0.01% control, individual per donor. **B** OCI-AML3 target cells or CD8+ T cells were pre-treated on day 1 and 4 with 10 nM TAK981 and/or 250 nM 5-Aza-2'. Subsequently, co-cultured with untreated OCI-AML3 or CD8+ NPM1-TCR T cells overnight. Supernatant was harvested and analysed by IFNγ ELISA. Three different donors were used for the generation of CD8+ NPM1-TCR T cells $P < 0.05$ = *, two-way ANOVA compared to DMSO 0.01%, followed by Fisher's LSD test, GraphPad Prism 9.3.1. **C** Experimental co-culture set up for CD8+ T cell survival upon TAK981 and/or 5-Aza-2' treatment, quantification by flow cytometry. **D** NPM1-TCR CD8+ T cell counts are shown upon 5 days of co-culture with irradiated OCI-AML3 cells (to prevent overgrowth). CD8+ T cells were treated with TAK981 at 10 nM and/or 5-Aza-2' at 250 nM or DMSO 0.01% control. Co-cultures were set up in T cell medium deficient of IL2. Data represent three different CD8+ T cell donor replicates. Each replicate is plotted individually as ratio to normalized DMSO control. **E** Extended data for Figure 3C and D. OCI-AML3 target cells or CD8+ T cells were pre-treated on day 1 and 4 with 10 nM TAK981 and/or 250 nM 5-Aza-2' or DMSO 0.01% as control. Subsequently, cells were co-cultured in all possible combinations of conditions. Supernatant was harvested and analysed by IFNγ ELISA. Five different donors were used for the generation of CD8+ NPM1-TCR T cells. **F** Heat map presentation of data from E.

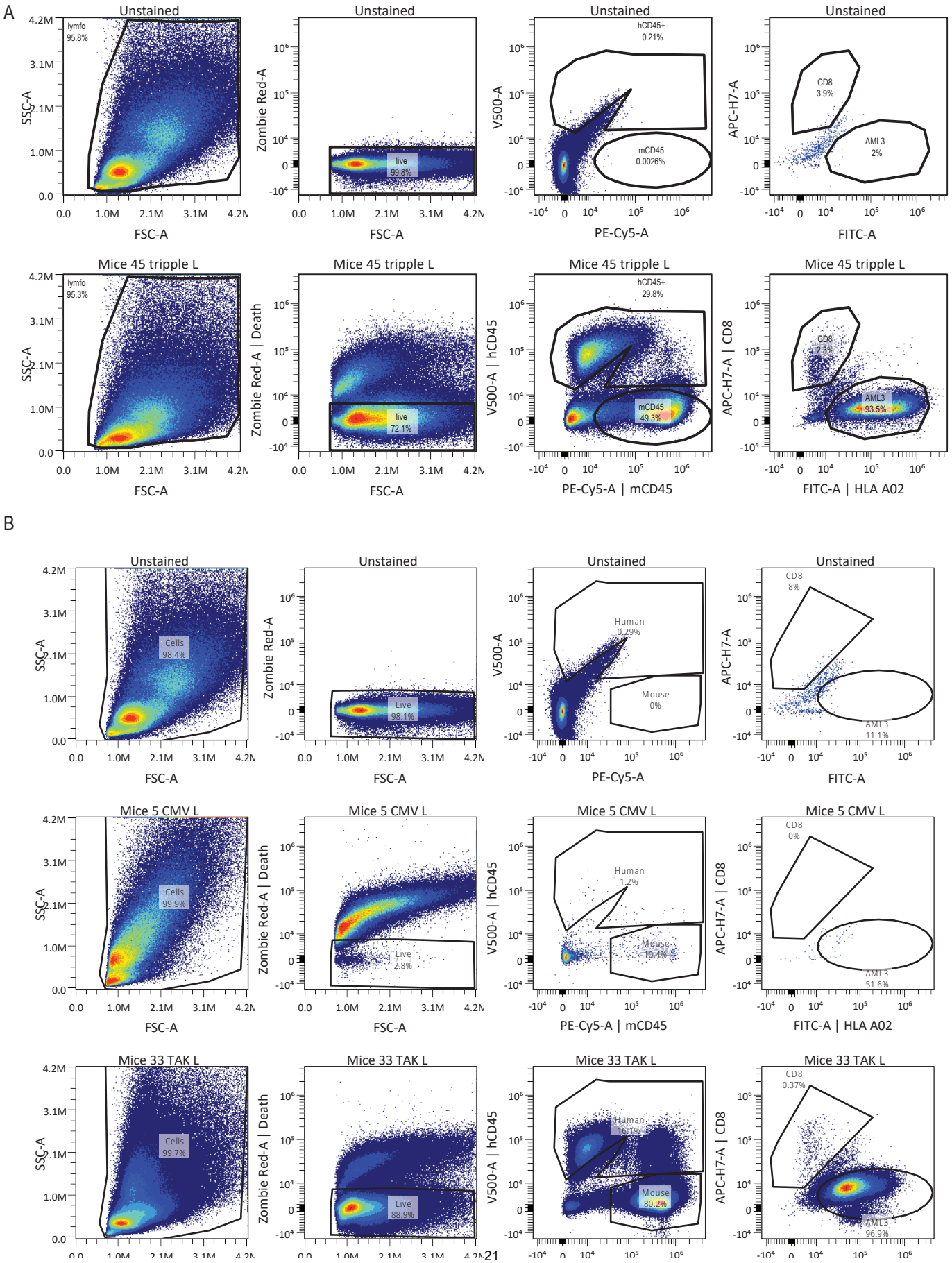

A
Unstained
lymfo 95.8%
SSC-A
FSC-A
Zombie Red-A
live 99.8%
V500-A
hCD45+ 0.21%
mCD45 0.0026%
PE-Cy5-A
APC-H7-A
CD8 3.9%
AML3 2%
FITC-A
Mice 45 tripple L
lymfo 95.3%
Zombie Red-A | Death
live 72.1%
V500-A | hCD45
hCD45+ 29.8%
mCD45 49.3%
PE-Cy5-A | mCD45
APC-H7-A | CD8
CD8 2.3%
AML3 93.5%
FITC-A | HLA A02
B
Cells 98.4%
Live 98.1%
Human 0.29%
Mouse 0%
CD8 8%
AML3 11.1%
Mice 5 CMV L
Cells 99.9%
Live 2.8%
Human 1.2%
Mouse 10.4%
CD8 0%
AML3 51.6%
Mice 33 TAK L
Cells 99.7%
Live 88.9%
Human 16.1%
Mouse 80.2%
CD8 0.37%
AML3 96.9%

Figure S5

Supplementary Figure 5 Gating strategy for data presented in Figure 4 D and E. **A** Control unstained sample from bone marrow harvested on Day 7 was used to set the gating. ZombieRed staining was used to gate live cells. Human cells were separated via differential mouse versus human CD45 staining. Human cells were gated and CD8+ cells were separated from OCI-AML3 cells via HLA-A2/HLA-ABC versus CD8+. One representative gating was shown for a mouse stained with indicated markers. **B** Similar to (**A**) gating for day 9 was performed. Extra row (middle) indicates an example for loss of live cells occurring in all the CMV-TCR samples and therefor were excluded from analysis. Samples were measured with Cytek Aurora spectral flow cytometer 3L (Cytekbio) and analyzed with OMIQ.ai (Dotmatics).

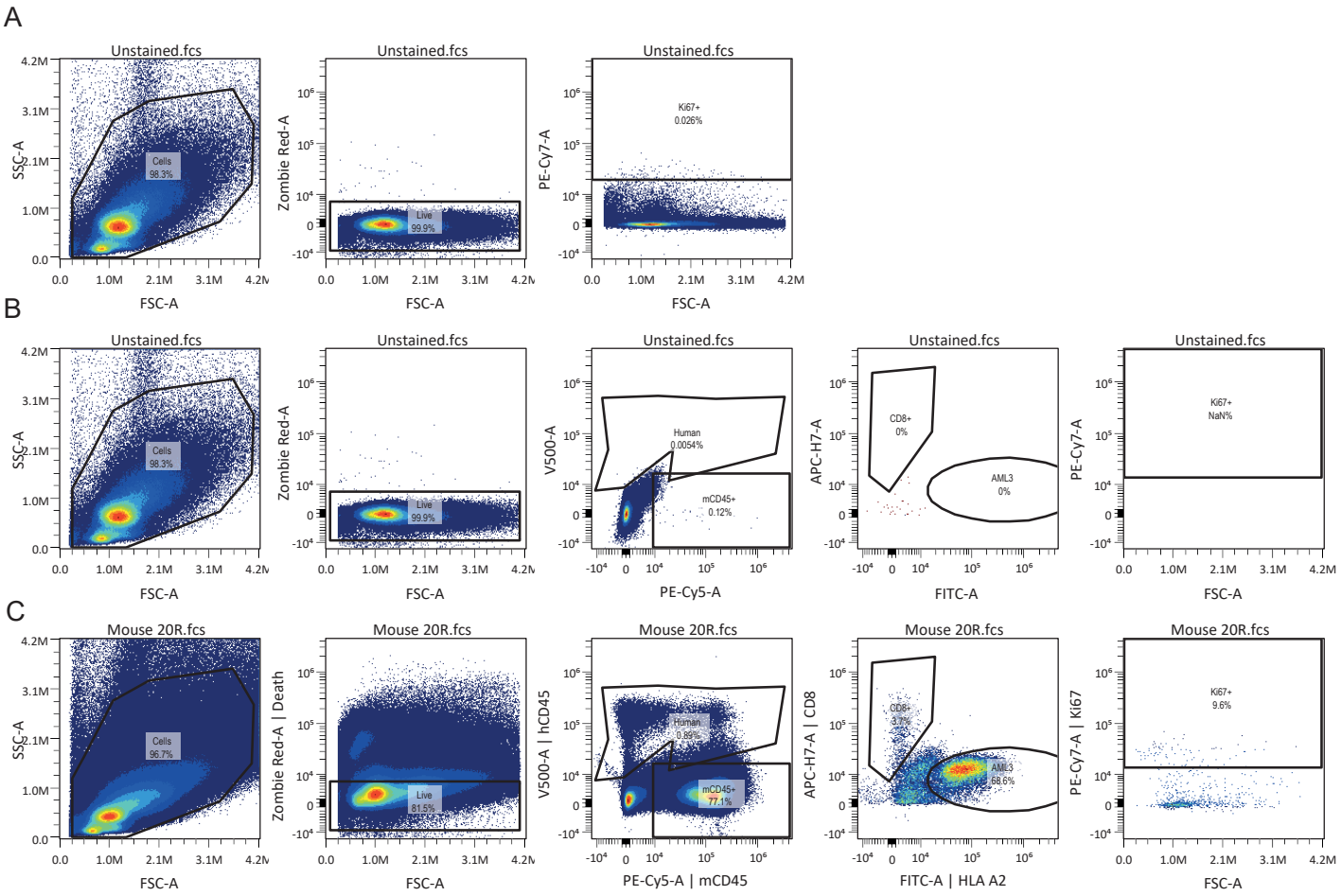

A
Unstained.fcs
SSC-A
FSC-A
Cells
98.3%
Zombie Red-A
Live
99.9%
PE-Cy7-A
Ki67+
0.026%
B
Unstained.fcs
V500-A
Human
0.0054%
mCD45+
0.12%
PE-Cy5-A
APC-H7-A
CD8+
0%
AML3
0%
FITC-A
Ki67+
NaN%
C
Mouse 20R.fcs
Cells
96.7%
Zombie Red-A | Death
Live
81.5%
V500-A | hCD45
Human
0.89%
mCD45+
77.1%
PE-Cy5-A | mCD45
APC-H7-A | CD8
CD8+
3.7%
AML3
68.6%
FITC-A | HLA A2
PE-Cy7-A | Ki67
Ki67+
9.6%

Figure S6

Supplementary Figure 6 Gating strategy for data presented in Figure 5 and 6. **A** Unstained sample was used to set the boundaries for the marker stainings. Example shown for Ki67 staining. **B** Control unstained sample was used to set the gating. ZombieRed staining was used to gate live cells. Human cells were separated via differential mouse versus human CD45 staining. Human cells were gated and CD8+ cells were separated from OCI-AML3 cells via HLA-A2/HLA-ABC versus CD8+. **C** Example sample indicating gating on stained sample. ZombieRed staining was used to gate live cells. Human cells were separated via differential mouse versus human CD45 staining. Human cells were gated and CD8+ cells were separated from OCI-AML3 cells via HLA-A2/HLA-ABC versus CD8+. For selected markers, mean fluor intensity (MFI) and percentage positive cells were analysed for CD8+ population and HLA-A2 or HLA-ABC populations. Samples were measured with Cytek Aurora spectral flow cytometer 3L (Cytekbio) and analyzed with OMIQ.ai (Dotmatics).