Supplemental Figure Legends:

Supplemental Figure 1: Testing the affinity of Nbs against variant AML cell lines.

(**A**) The four THP-1 specific Nbs were tested for their recognition to other AML cell lines. + was <0.5 log shift, ++ was <1.0 log shift, +++ was < 1.5 log shift, - was no binding. (N=3).

Supplemental Figure 2: The expression of the Nb CARs protein in human primary T cells

(A) Western blot with anti-human CD3z to show the Nb CAR expression in primary T cells transduced by the lentivirus. Beta-mercaptoethanol (BME) is suitable for reducing protein disulfide bonds, to make the dimer protein to monomer.

(B) Flow cytometry showed GFP expression in primary T cells transduced with Nb CAR lentivirus. (N=3).

Supplemental Figure 3: Nb176 and Nb393 CAR T cells display potent suppression against THP-1 tumor *in vitro* and *in vivo*.

(**A**-**B**) Nb176 and Nb393 CARs showed potent and specific cytotoxicity against THP-1 cells in a dose dependent manner, but not to K562 cells. Untransduced (UTD) T cells do not exert obvious cytotoxicity. (N=3, One-way ANOVA: *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ns, $p > 0.05$).

(C) HL60 cells, not Jurkat cell, stimulated Nb157 CAR T cell to secrete IFN-gamma. Primary T cells were activated and transduced by the Nb157 CAR lentivirus, followed by incubation with HL60 cells or Jurkat cells in different E/T ratios. 16 hour later, IFN-gamma in the supernatant was detected by ELISA. (N=3, student *t* test, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ns, $p > 0.05$).

(D) 5 million K562 cells were transplanted into NSG mice subcutaneously. The tumor reached 150 mm^3 after about 14 days. 3 million Nb157, Nb163 CAR or UTD T cells were injected intravenously into NSG mice separately. The tumor engraftments were monitored every other day. 9 days after T cells treatment, the K562 tumor tissues were harvested and recorded. Scale bar was 10 mm. (N=4).

(E) Hematoxylin and eosin stain of K562 xenograft after Nb157, Nb163 CAR or untransduced T cell treatment. Scale bar was 200 um.

(**F-G**) 10 million THP-1 cells were transplanted into NSG mice subcutaneously. The tumor reached 150 mm^3 after about 14 days. 3 million Nb176, Nb393 CAR or UTD T cells were injected intravenously into NSG mice separately. The tumor engraftments were monitored every other day. Scale bar was 10 mm. (N=3, One-way ANOVA: *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ns, $p > 0.05$).

(**H**) Hematoxylin and eosin stain of THP-1 xenograft after Nb176, Nb393 CAR or untransduced T cell treatment. Scale bar was 100 um. (N=4).

Supplemental Figure 4: Nb157 CAR T cells eradicate patient-derived AML in NSG mouse model.

(**A**) Body weight of mice with PD-AML and UTD or CAR T cells was monitored weekly. (N=10).

(**B-C**) PD-AML and Nb CAR T cells in mouse peripheral blood were monitored weekly by staining with anti-human CD45/CD33 or CD45/CD3. In the B panel, Yellow line indicated the superpositions of the two groups (combination of green and red), which were then divided into

the two groups randomly. (N=3, student *t* test, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ns, $p > 0.05$).

(D) CAR positive T cells in mouse bone marrow and spleen were detected by tracking GFP from the hCD45+/hCD3+/hCD33- subset two weeks after T cells infusion. (N=3).

(E) The CD4 and CD8 percentages in total hCD3 T cells from bone marrow 3 weeks post T cell infusion were analyzed by flow cytometry. (N=3).

(F) Memory T cells in mouse peripheral blood were detected 3 weeks after T cells infusion. CD45RA-CD62L+ are the central memory T cells, CD45RA-CD62L- are the effector memory T cells. (N=3 and Student *t* test was applied, *, $p<0.05$).

Supplemental Figure 5: TIM3 CAR redirects T cells to eliminate the AML cells

(A) TIM3 expression in NB4-TIM3 was confirmed by western blot. anti-TIM3 antibody was applied. Star indicated the potential glycosylation-modified TIM3 protein.

(B) TIM3 and CD13 expressions in NB4 or NB4-TIM3 were detected by flow cytometry. (N=3).

(C) Schematic diagram of conventional second generation of TIM3 CAR construct. An anti-TIM3 scFv was linked with 4-1BB and CD3z domains.

(D) TIM3 CAR showed potent and specific cytotoxicity against NB4-TIM3 cells in a dose dependent manner, but not to NB4 cells. Untransduced (UTD) T cells did not exert obvious killing. (N=3, One-way ANOVA: *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ns, $p > 0.05$).

(E) TIM3 CAR T cells suppressed, but did not eradicate the NB4-TIM3 tumor *in vivo*. 10 million NB4-TIM3 cells were transplanted into NSG mice subcutaneously. The tumor reached 150 mm^3

after about 10 days. 3 million TIM3-BBz CAR or UTD T cells were injected into NSG mice separately. The tumor engraftments were measured and monitored at the indicated time (N=3).

(**F-G**) Schematic diagram of Nb157 CAR and BissCAR. Both constructs were packaged into lentivirus to infect the activated human primary T cells. Nb157 CAR and anti-TIM3 scFv expressions were detected by flow cytometry. The Nb157 and anti-TIM3 of BissCAR on T cell surface were detected by the homemade Rabbit-anti-Nanobody and Biotin-conjugated anti-mouse scFv, respectively, followed by staining with Donkey-anti-Rabblit-AF647 and Streptavidin-PE. (N=3).

(**H**) Both Nb157 CAR and BissCAR T cells showed potent cytotoxicity against NB4 and NB4-TIM3 cells in a dose dependent manner. Untransduced (UTD) T cells did not exert obvious killing. (N=3, One-way ANOVA: *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ns, $p > 0.05$).

(**I-J**) NB4, NB4-TIM3 and K562-TIM3 cells were incubated with BissCAR or UTD T cells at a ratio of 1:1 between Effector to Target at 37 degree for 16 hours. The cytokines including TNFα and IFNγ in the media supernatant were detected by ELISA. (N=3, student *t* test, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ns, $p > 0.05$).

Supplemental Figure 6: Analysis of the PD-1, TIM3, Treg T cells in the bone marrow and spleen from the BissCAR or UTD T cells treated PD-AML mice

(**A-D**) Representative gating for the flow cytometry analysis of PD-1 and TIM3 in the hCD3+CD8+ subset from mice bone marrow or spleen cells 3 weeks post T cell infusion.

(**E-F**) Proportion of PD-1 or TIM3 cells among the hCD3+CD8+ subset from mice bone marrow or spleen cells 3 weeks post T cell infusion. (N=3, student *t* test, *, $p<0.05$, ns, $p > 0.05$).

(**G-J**) Representative gating for the flow cytometry analysis of Treg (Foxp3+CD25) in the hCD3+CD4+ subset from mice bone marrow or spleen cells 3 weeks post T cell infusion.

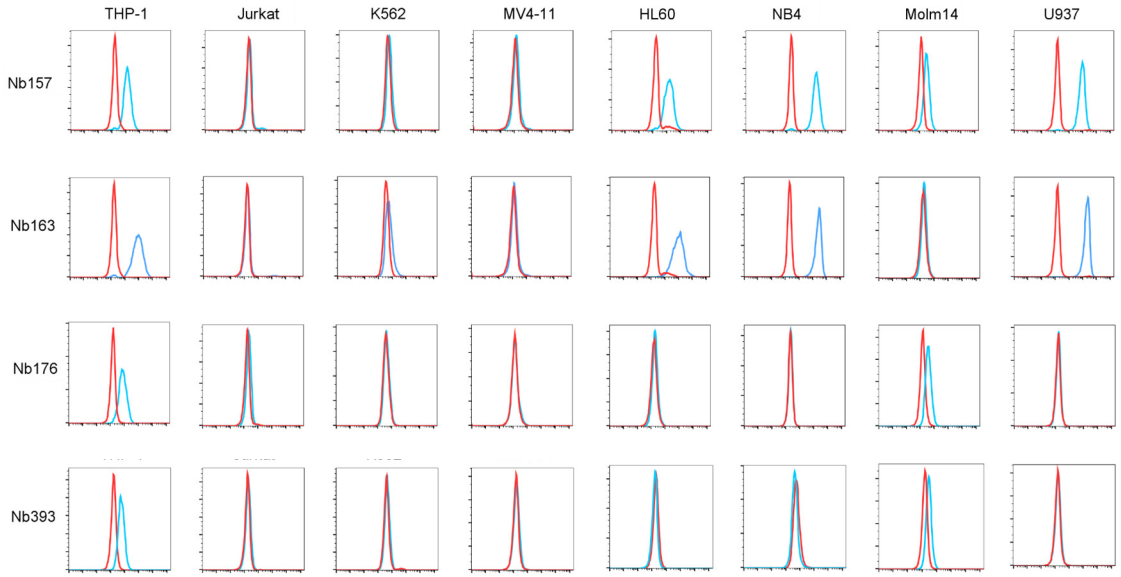
(**K**) Proportion of Treg cells among the hCD3+CD4+ subset from mice bone marrow or spleen cells 3 weeks post T cell infusion. (N=3, student *t* test, ns, $p > 0.05$).

Supplemental Figure 7: The information of the patient-derived AML

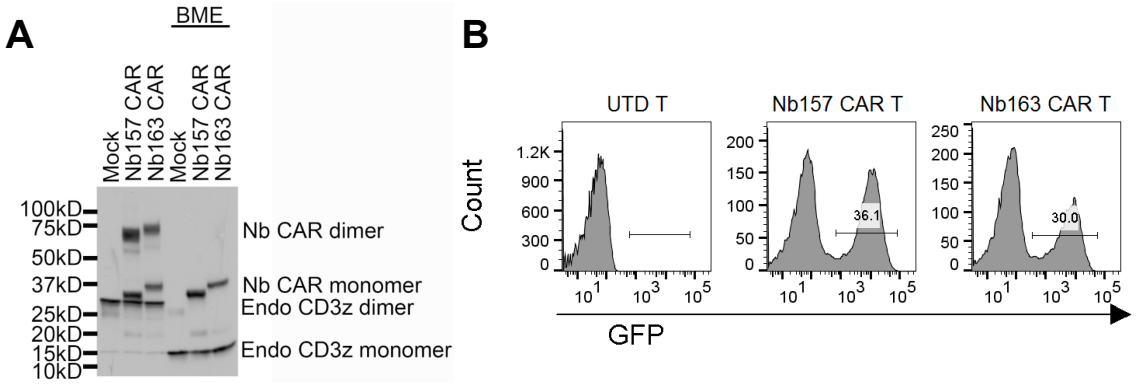
Supplemental Figure 1

A

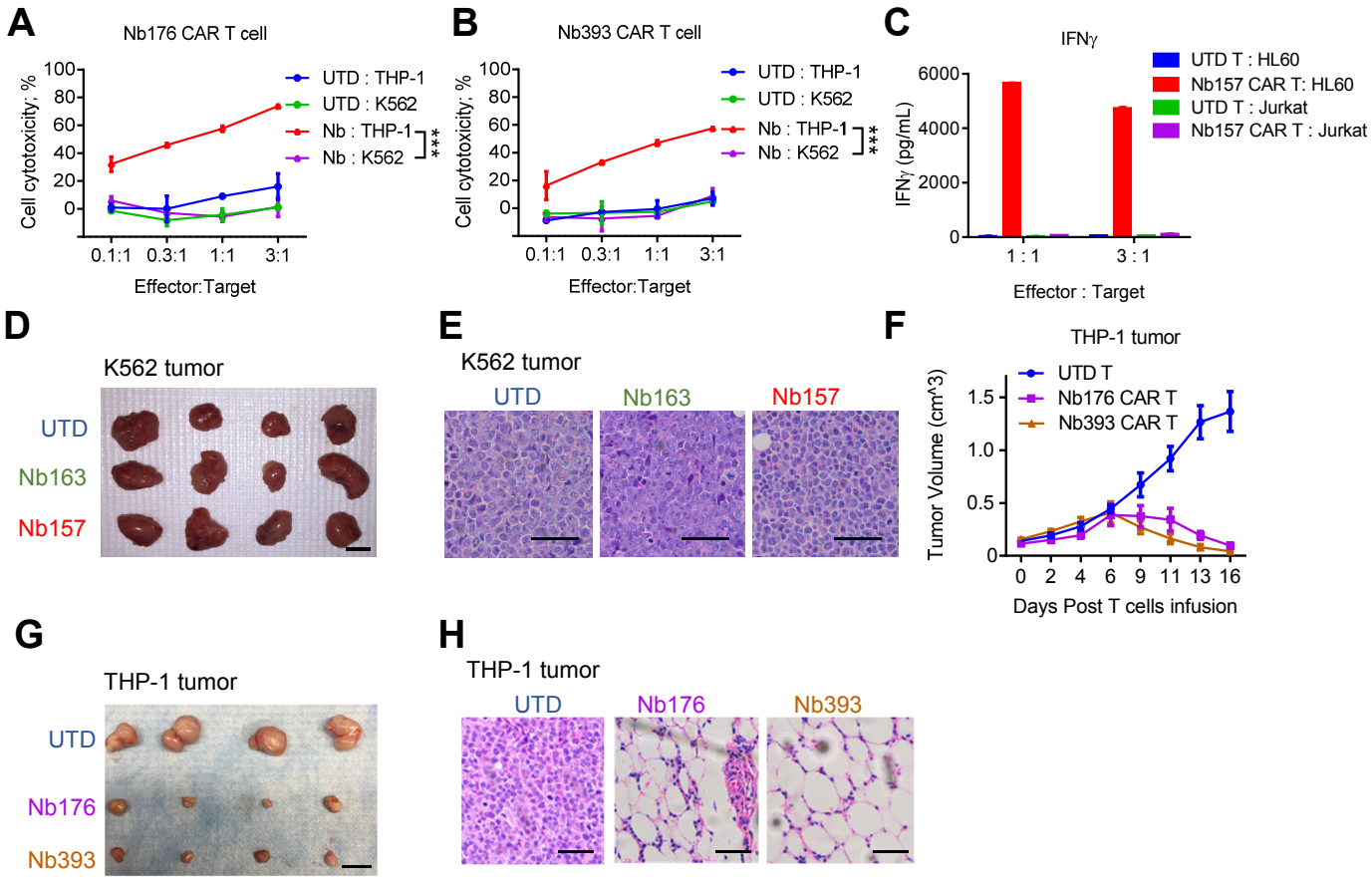
	AML (M5)	T cell Leukemia	CML	AML (M2)	AML (M3)	AML (M4)	AML (M5)	AML (M5)
	THP-1	Jurkat	K562	HL60	NB4	MV4;11	Molm14	U937
Nb157	++	-	-	+	+++	-	+	+++
Nb163	+++	-	-	++	+++	-	-	+++
Nb176	++	-	-	-	-	-	+	-
Nb393	+	-	-	-	-	-	+	-



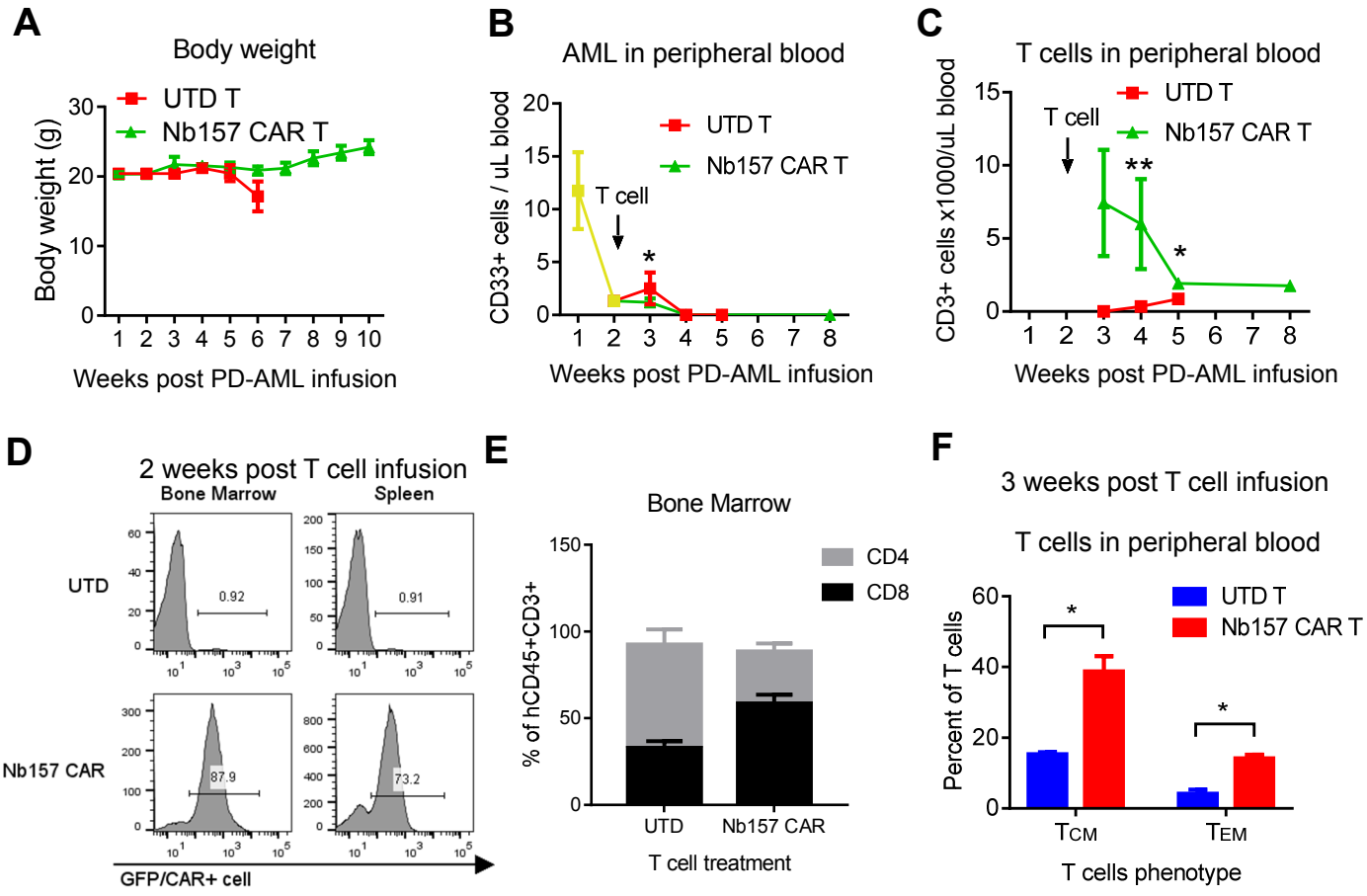
Supplemental Figure 2

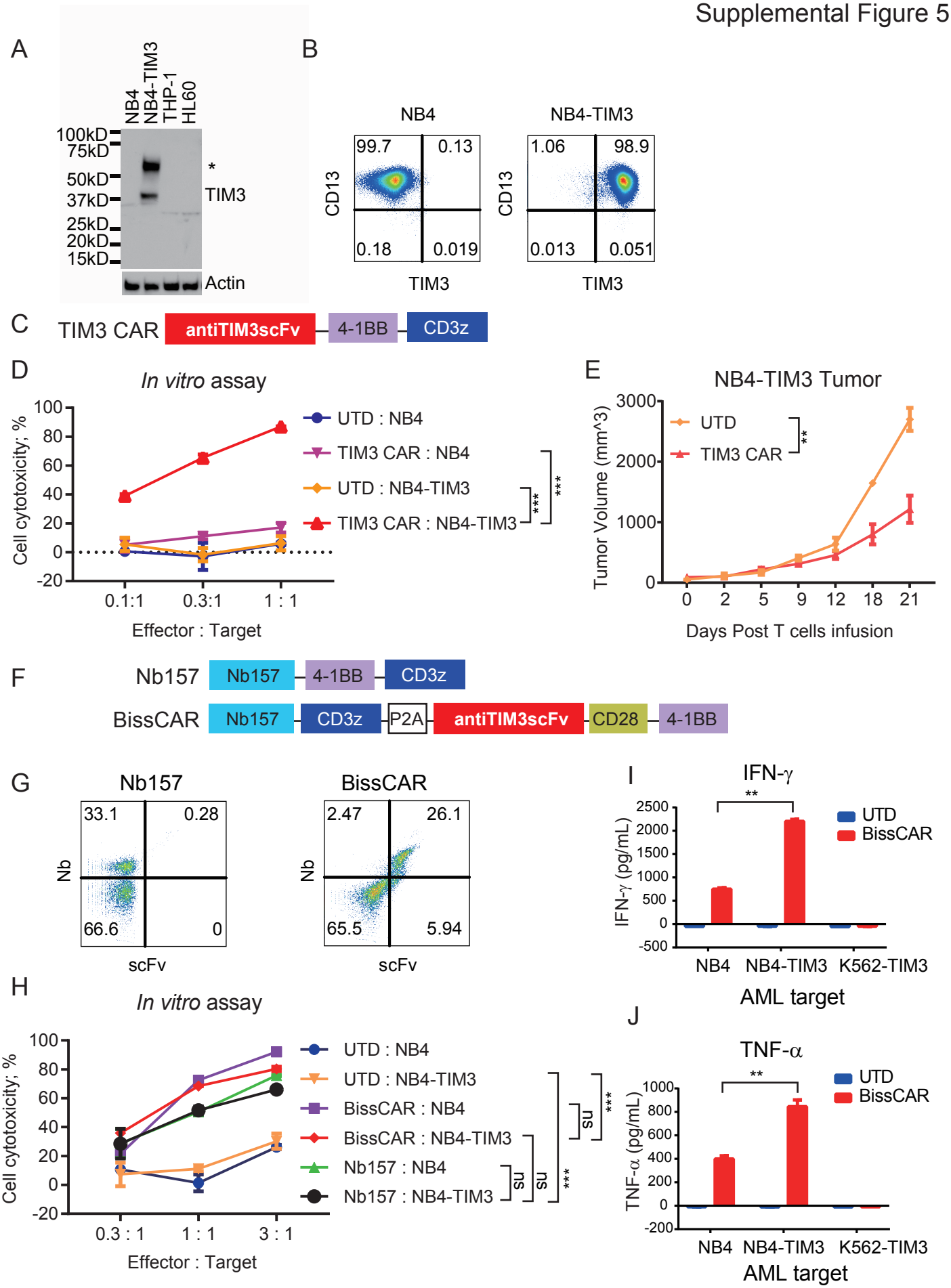


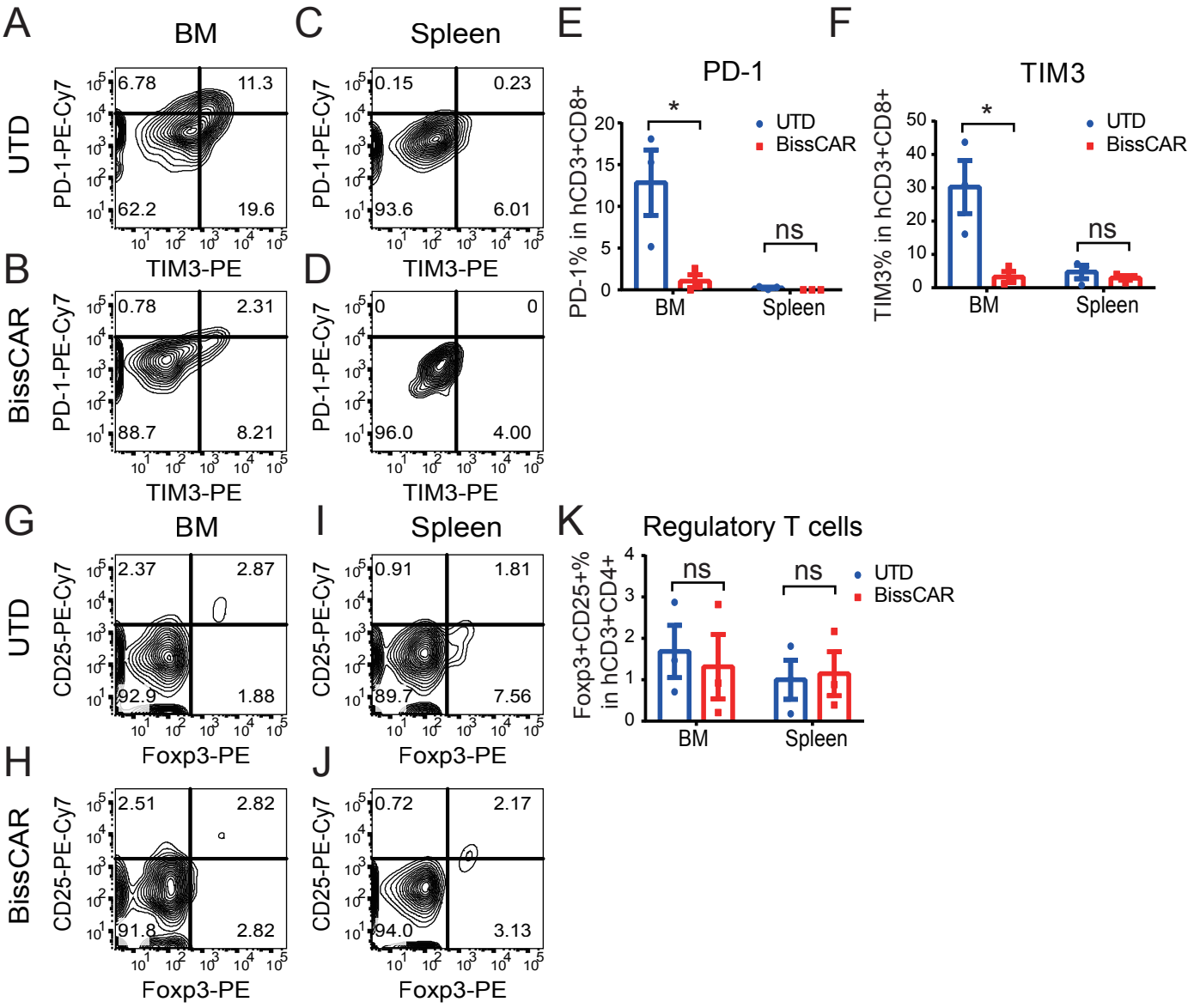
Supplemental Figure 3



Supplemental Figure 4




A
NB4
NB4-TIM3
THP-1
HL60
100kD
75kD
50kD
37kD
25kD
20kD
15kD
*
TIM3
Actin
B
NB4
NB4-TIM3
CD13
TIM3
99.7
0.13
0.18
0.019
1.06
98.9
0.013
0.051
C
TIM3 CAR
antiTIM3scFv
4-1BB
CD3z
D
In vitro assay
Cell cytotoxicity; %
Effector : Target
0.1:1
0.3:1
1 : 1
UTD : NB4
TIM3 CAR : NB4
UTD : NB4-TIM3
TIM3 CAR : NB4-TIM3
***
***
E
NB4-TIM3 Tumor
Tumor Volume (mm^3)
Days Post T cells infusion
UTD
TIM3 CAR
**
F
Nb157
BissCAR
Nb157
4-1BB
CD3z
CD28
P2A
antiTIM3scFv
G
Nb157
BissCAR
Nb
scFv
33.1
0.28
66.6
0
2.47
26.1
65.5
5.94
H
In vitro assay
Cell cytotoxicity; %
Effector : Target
0.3 : 1
1 : 1
3 : 1
UTD : NB4
UTD : NB4-TIM3
BissCAR : NB4
BissCAR : NB4-TIM3
Nb157 : NB4
Nb157 : NB4-TIM3
ns
ns
ns
***
***
I
IFN-γ
IFN-γ (pg/mL)
**
UTD
BissCAR
NB4
NB4-TIM3
K562-TIM3
AML target
J
TNF-α
TNF-α (pg/mL)
**
UTD
BissCAR
NB4
NB4-TIM3
K562-TIM3
AML target


A
BM
UTD
PD-1-PE-Cy7
6.78
11.3
62.2
19.6
TIM3-PE
B
BissCAR
0.78
2.31
88.7
8.21
C
Spleen
0.15
0.23
93.6
6.01
D
0
0
96.0
4.00
E
PD-1
PD-1% in hCD3+CD8+
UTD
BissCAR
*
ns
BM
Spleen
F
TIM3
TIM3% in hCD3+CD8+
G
CD25-PE-Cy7
2.37
2.87
92.9
1.88
Foxp3-PE
H
2.51
2.82
91.8
2.82
I
0.91
1.81
89.7
7.56
J
0.72
2.17
94.0
3.13
K
Regulatory T cells
Foxp3+CD25+% in hCD3+CD4+

Supplemental Figure 7

Collection_ ID	Diagnosis	Subtype	Status_ Collection	Treatment	Cytogenetics_ Abnormality	All_Flow Results_List
2844	AML	M5 (combining both M5a and M5b)	De Novo	None	45,X,-Y[19]/46,XY[1]	CD5- CD10- CD13+ CD14 var + CD15+ CD19- CD20- CD33+ CD56 var + CD64+ CD79- HLA-DR+ TdT-

Supplemental Methods

Cell Lines, Cell Culture, Plasmids and Antibodies. THP-1, K562, NB4, HL60, Jurkat, U937, MV4-11 cell lines were obtained from the ATCC and maintained in RPMI1640 with 10% FBS and 1% penicillin/streptomycin (R10 medium) and maintained at 37C and 5% CO_2. HEK293T cells were obtained from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Deidentified Patient derived AML cells were obtained from the University of Pennsylvania Stem Cell and Xenograft Core facility and maintained in R10 medium. Normal donor total T cells were obtained from the Human Immunology Core at University of Pennsylvania, and maintained in the R10 medium.

pComb3XSS was a gift from Carlos Barbas (Addgene plasmid # 63890). pHIV-EGFP was a gift from Bryan Welm & Zena Werb (Addgene plasmid # 21373). lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961). A human membrane protein cDNA library was provided by High-throughput Screen Core at University of Pennsylvania.

Nanobody phage library construction from the THP-1 cell-immunized llama. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of immunized llama through Ficoll gradient centrifugation (GE). Total RNA was extracted by the RNeasy Mini Kit (QIAGEN) as the manufacturer's instructions, followed by cDNA synthesis from SuperScript™ III First-Strand Synthesis System (Invitrogen).

Molecular clone for nanobody phage library construction. Nanobody encoding fragments were amplified from llama PBMC cDNA using the method described by Vincke et al.,(1) modified by the use of PCR primers with SfiI sites designed to be compatible with cloning into the pComb3X phage display vector.(2) The resulting phage library was generated by infection

with VCSM13 helper phage and PEG-precipitated from the bacterial supernatant in preparation for cell-surface selection,(2) followed by panning and identification.

A 1st round of PCR using primers Forward 5'- GTCCTGGCTGCTCTTCTACAAGG and Backward 5'-GGTACGTGCTGTTGAACTGTTCC yielded a larger conventional heavy chain band (VH-CH1-CH2) and smaller heavy chain band lacking CH1 (VH-CH2). The smaller band was gel purified and used as template for a second round of PCR with primers Forward 5'-GAGGAGGAGGAGGAGGAGGCGG*GGCCCAGGCGGCCC*AGGTGCAGCTGCAGGAGTC TGGRGGAGG and Backward 5'-GAGGAGGAGGAGGAGGAGCCT*GGCCGGCCTGGCC*ACTAGTGGCGGCCGCTGAGGA GACGGTGACCTGGGT where the appended SfiI sites are underlined. Following gel purification and digestion with SfiI, nanobody amplified DNA segments were ligated into SfiI-digested phagemid pComb3X. The ligation product was electroporated into competent cells, yielding ~10^9 independent transformants.

Nanobody phage library panning. The titer of concentrated phage was generally 1×10^{13} to 1×10^{14} cfu/mL. In the first round of panning, the phage was applied to bind THP-1 directly, followed by washing, acidic elution (0.2 M Glycine-HCl pH 2.2) and neutralization, to enrich the tumor specific Nb-expressing phage (3). In the second round of panning, the elution of phage was amplified and concentrated to bind Jurkat cells as the negative selection. The unbound phage from Jurkat cells were incubated with THP-1 cells to enrich and then eluted with acidic elution. In the third round of panning, K562 was used for negative selection.

Construction of nanobody CAR vector. Generation of the Nanobody CAR (Nb CAR) constructs in lentiviral vector was shown in **Fig. 2A**. In brief, the plasmid backbone is pHIV-eGFP, a third-generation self-inactivating lentiviral vector plasmid. Nb CAR constructs,

including CD8 signal peptide (SP), SfiI cut sites, IgG4m hinge, 4-1BB domain and CD3z domain, was custom synthesized by IDT-DNA. IRES-GFP was used as a marker to track Nb CAR positive cells. Nb CAR expression was confirmed by Western blots using anti-CD3z (**Fig. S2A**) (Abcam). Nanobody fragments were cloned into in-frame from pComb3XSS into pHIV-eGFP-CAR at the two sequential SfiI sites.

***In vivo* tumor-mediated selection of nanobodies from CAR T cells.** Nanobody cDNA isolated from phage particles following cell-based selection were amplified with PCR using primers 2nd round Forward and Backward, followed by SfiI digestion and ligation into pHIV-eGFP-CAR vector. The ligation product was electroporated into competent cells, yielding at least 2 x 10^6 independent clones in transformation, followed by lentivirus packaging and infecting activated human primary T cells to generate Nb-lib-CAR-expressing T cells. The resulting T cells (> 2 x 10^7, with >20% GFP+) or untransduced (UTD) T cell were administered intravenously into NSG mice bearing either THP-1 tumor or K562 tumor, as shown in the **Fig. 1B**. Fourteen days later, cDNAs for nanobodies were amplified by PCR from the genomic DNA isolated from the tumor infiltrated Nb-lib-CAR T cells, with the primer Nb-Amp-Forward, 5'-ATTTCAGGTGTCGTGAGCGG; and Nb-Amp-Backward, 5'-AGGAGAAGGACCCCACAAGT. The PCR product was digested with SfiI and inserted into pComb-3XSS, followed by randomly picking of individual clones and sequencing with primer Nb-Seq-Forward, 5-CAGCTATCGCGATTGCAGT.

***In vitro* analysis of T cell function.**

T-cell transduction. HEK293T cells were co-transfected with lenti-vector plasmid, psPAX2 and VSV-G plasmids DNA to produce the lentivirus 48h after transfection. Normal donor T cells were positively selected from leukapheresis packs using anti-CD4 and CD8 microbeads

(Miltenyi), expanded *in vitro* with anti-CD3/CD28 beads (Invitrogen) for up to 12 days. Total T cells were transduced with lentiviral 24 hours after activation. The resulting virus from the supernatant were concentrated via untracentrifuation at 25,000 g for 2.5 h at 4C.

Killing assays were performed as previously described (4). In brief, target cells were labeled by anti-CD33 (BD) for detecting cell number with flow cytometry analysis or labeled by CellTrace Far Red for tracing cell division. Target cells were incubated with effector T cells for 16 hrs at a series of ratios. Cells were then harvested, washed, and stained by propidium iodide prior to flow cytometry analysis. Quantification were calculated by either Countbright beads or volume.

To detect cytokine secretion, effector and target cells were incubated at a 1:1 ratio in R10 medium for 16 hrs as indicated. Supernatant was analyzed using Human TNF-alpha or IFN-gamma DuoSet ELISA kits according to the manufacturer's instructions (R&D System).

T cell degranulation assay, activated and Nb CAR transduced or untransduced T cells (1×10^5 cells) were co-cultured with THP-1 or K526 cells at a 1:1 ratio in 96-well plates for 4 hrs, in the presence of APC-conjugated anti-CD107a antibody, followed by wash and flow cytometry analysis.

T cell proliferation assay, T cells were labeled by CellTrace™ Far Red Cell Proliferation Kit (Invitrogen) as the manufacturer's instructions. The reaction was quenched with R10 medium, and the cells were washed twice. T cells were incubated at a 1:1 ratio with heat-inactivated target cells for 96 hrs.

Rabbit-anti- Nanobody polyclonal Antibody. Rabbit-anti-VHH were contracted with and made by the Covance company (Covance, Inc. PA US). A mixer of four different VHH protein, purified by Ni-NTA affinity from *E.coli,* were utilized to immunize the Rabbit four times,

followed by serum purification by the Protein A/G affinity method. The immunization response was monitored by the ELISA experiment. The purified serum was titrated and analyzed by flow cytometry, followed by the staining with Donkey-anti-Rabbit-AF647 (Cat. 406414, BioLegend).

THP-1 CD13 knockout cell line. To knockout CD13 in THP-1 cell line, LentiCRISPRv2.0 vector was applied according to the protocol (5). sgRNA targeting human CD13 were listed as below, sgCD13-1 ATGGCCGGCTCATCGAAGCA, sgCD13-2 CTTCCCATGCTTCGATGAGC, sgCD13-2 CTTCATGGGGCCATAGACCT. Plasmids were confirmed by sequencing and packaged into lentivirus, followed by infecting THP-1 cells. Single clones were randomly picked up from each group after puromycin seletion (2.5 ug/mL) for 4 days.

Animals and *in vivo* models.

All laboratory mice were maintained on a 12-hr light-dark cycle in the animal facility at the University of Pennsylvania. All experiments on mice in our research protocol were approved by Institutional Animal Care and Use Committee of the University of Pennsylvania and were performed in accordance with relevant institutional and national guidelines and regulations.

NOD/Shi-scid/IL-2Rγnull (NSG) mice, 8-12 weeks old, were obtained from Jackson Laboratories. NSG mice were inoculated with 1×10^7 cells of THP-1, HL60 or NB4 subcutaneously, or with 0.5×10^7 K562 cells subcutaneously. When tumor volume reached 150 mm^3 around 12 days after xenografting, Nb CAR T cells or untransduced (UTD) human T cells (1×10^7 cells) were administered via tail vein. Mice and tumors were monitored every other day. Tumor dimensions were measured with Vernier calipers and tumor volume was calculated as 1/2 larger diameter × (smaller diameter)^2.

NSG mice were conditioned by Busulfex (30 mg/kg) 24 hrs prior to tail injection with $2x10^5$ of bone marrow-derived CD34+ from normal donor. Four weeks later, CAR or UTD T cells were transduced into the mice. The recipient mice were sacrificed three weeks after initial treatment, and the long bones (femurs) and spleens were collected for histological analysis by H&E staining.

Statistical Analysis. Microsoft Excel and GraphPad Prism software were used for statistical analysis. Student's t test was used to determine the significance of the results unless otherwise indicated. Kaplan-Meier statistical analysis was performed using the log rank test. In the figures, asterisks denote statistically significant p values (*, $p<0.05$, **, $p<0.01$, ***, $p<0.001$), and "ns" indicates lack of statistical significance ($p > 0.05$). All error bars are represented either SEM or SD.

Supplemental Material Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-CD13	Abcam	Cat# ab227111
anti-M13/fd/F1 Filamentous Phages Biotin mouse monoclonal, B62-FE2	PROGEN	Cat# 61597
APC Mouse Anti-Human CD107a	BD Biosciences	Cat# 560664
hCD45-V450	BD Biosciences	Cat# 560368
PerCP-Cy™5.5 Mouse Anti-Human CD3	BD Biosciences	Cat# 560835
Anti-Human CD33 APC	eBioscience	Cat# 17-0338-41
PE-Cy™7 Human CD45RA	BD Biosciences	Cat# 560675
BV 421™ human CD62L	BioLegend	Cat# 304827
Fc Blocker	BD Biosciences	Cat# 564220
Anti-CD3 zeta antibody	Abcam	Cat# ab226263
Bacterial and Virus Strains		
ER2738 Electrocompetent Cells	Lucigen	Cat# 60522
Endura™ ElectroCompetent Cells	Lucigen	Cat# 60242
one shot STBL3 competent cells	Thermo Fisher	Cat# C737303
One Shot TOP10 Chemically Competent	Thermo Fisher	Cat# C404003
Biological Samples		
Normal human T cells	Human Immunology Core at UPenn	N/A
Patient-derived AML	Stem Cell and Xenograft Core at UPenn	N/A
Chemicals, Peptides, and Recombinant Proteins		
IL-2	Gibco	Cat# PHC0026
IPTG	Fisher	Cat# BP162010
FuGENE® 6	Promega	Cat# E2691
Imidazole	Sigma-Aldrich	Cat# I5513
Critical Commercial Assays		
DuoSet ELISA Ancillary Reagent Kit 2	R&D Systems	Cat# DY008
Human TNF-alpha DuoSet ELISA	R&D Systems	Cat# DY210-05
Human IFN-gamma DuoSet ELISA	R&D Systems	Cat# DY285B-05
CountBright; absolute counting beads	Thermo Fisher	Cat# C36950
Compensation Particles Set	BD Biosciences	Cat# 552843
ArC™ Amine Reactive	Thermo Fisher	Cat# A10628
Dynabeads Human T-Activator CD3/CD28	Thermo Fisher	Cat# 11131D
Experimental Models: Cell Lines		
THP-1	ATCC	Cat# TIB-202

Jurkat, Clone E6-1	ATCC	Cat# TIB-152
K562	ATCC	Cat# CCL-243
HL60	ATCC	Cat# CCL-240
U937	ATCC	Cat# CRL-1593.2
THP-1 CD13 ko-1	This paper	N/A
THP-1 CD13 ko-2	This paper	N/A
MV4-11	ATCC	Cat# CRL-9591
HEK293T	ATCC	Cat# CRL-3216
Experimental Models: Organisms/Strains		
NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (6-8 weeks)	Jackson Laboratory	Cat# 005557
Oligonucleotides		
Primers for 1st round PCR of llama Nb amplification	FORWARD: GTCCTGGCTGCTCTTCTACAAGG	BACKWARD: GGTACGTGCTGTTGAACTGTTCC
Primers for 2nd round PCR of llama Nb amplification	FORWARD: GAGGAGGAGGAGGAGGAGGCGG *GGCCCAGGCGGCC*CAGGTGCAGCTGCAGGAGTCTGGRGGAGG	BACKWARD: GAGGAGGAGGAGGAGGAGCCT *GGCCGGCCTGGCC*ACTAGTGGC
Primers for PCR of tumor-infiltrated Nb amplification	FORWARD: ATTTCAGGTGTCGTGAGCGG	BACKWARD: AGGAGAAGGACCCCACAAGT
Primer for Sequencing tumor-infiltrated Nb	CAGCTATCGCGATTGCAGT	
sgRNA targeting sequence: hCD13 #1: GCTCATGTTTGACCGCTCCG	PRIMER FORWARD: CACCGGCTCATGTTTGACCGCTCCG	PRIMER BACKWARD: AAACCGGAGCGGTCAAACATGAGCC
sgRNA targeting sequence: hCD13 #2: ATGGCCGGCTCATCGAAGCA	PRIMER FORWARD: CACCGATGGCCGGCTCATCGAAGCA	PRIMER BACKWARD: AAACTGCTTCGATGAGCCGGCCATC
sgRNA targeting sequence: hCD13 #3: CTTCATGGGGCCATAGACCT	PRIMER FORWARD: CACCGCTTCATGGGGCCATAGACCT	PRIMER BACKWARD: AAACAGGTCTATGGCCCCATGAAGC

Recombinant DNA		
pComb3XSS	Carlos Barbas	Addgene Plasmid #63890
pHIV-EGFP	Bryan Welm & Zena Werb	Addgene Plasmid #21373
lentiCRISPRv2	Feng Zhang	Addgene Plasmid #52961
pHIV-EGFP-Nb-CAR	This paper	N/A
Software and Algorithms		
Graph Pad Prism 5	Graph Pad	N/A
FlowJo V10	TreeStar	N/A
Other		
LIVE/DEAD® Yellow	Thermo Fisher	Cat# L34967
LIVE/DEAD® Fixable Far Red	Thermo Fisher	Cat# L34973
LIVE/DEAD® AQUA	Thermo Fisher	Cat# L34965
CellTrace™ CFSE Cell Proliferation Kit	Thermo Fisher	Cat# C34570
CellTrace™ Far Red Cell Proliferation Kit	Thermo Fisher	Cat# C34572

Reference

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Worksheet: Match Criteria for Human Cell Line Authentication

Background

Cell lines may undergo genetic drift with passage. This can be minimised through good cell culture practice – for example, by returning to early passage frozen stocks rather than passaging a cell line for prolonged periods. However, even with good cell culture practice, genetic drift is known to occur in some cell lines. This may result in differences when comparing STR profiles from the same donor.

A Standard was recently published on authentication of human cell lines by STR profiling (1). The Standard recommends a set of match criteria, allowing for some degree of genetic drift when interpreting STR profile results from human cell lines. Recommendations in the Standard are supported by more than ten years of publications in this area (2,3).

This worksheet will help you apply the match criteria recommended in the Standard to your sample.

Step 1: Obtain an STR Profile for your Test Sample

Refer to the Standard for more information on authenticating human cell lines. The quality of the STR profile produced is particularly important for your Test Sample to be interpreted correctly (1). Kits can be purchased by laboratories who wish to develop their own STR profiling. Authentication testing is also performed commercially by various suppliers, including several of the cell repositories.

Step 2: Identify a Reference Sample for Comparison

To interpret an STR profile result, you must compare to other samples. DNA from the same donor is the best comparison, if available, showing whether the cell line comes from the same donor. If donor DNA is not available, you will need to compare your sample to a range of other cell lines to demonstrate that cross-contamination is unlikely to have occurred. Online interactive databases of STR profiles from human cell lines are currently available for comparison via the ATCC and DSMZ websites. These databases (which require login access) will compare your sample to all available STR profiles (Step 3) and return a list of closest matches.

The "Reference Sample" referred to in this worksheet is defined as a previously authenticated sample, coming from any of these sources.

Step 3: Compare Test and Reference Samples using the Match Criteria given

3a) Enter your STR profile results into Table 3A on the following page

The table normally has space for the eight core STR loci (plus amelogenin for gender determination) recommended as the minimum for authenticating human cell lines (1). If test results have been obtained using a greater number of loci, add additional rows to the table to include all loci tested.

Normally either one or two alleles are amplified at each locus. Two alleles are indicated by two numbers separated by a comma e.g. 7,8. Three or more alleles at some loci are consistent with a mixture, although this finding can occur in the absence of a mixture in a small number of cell lines. Compare to the reference profile before you conclude whether a mixture is present.

3b) Perform the calculations listed in Table 3B on the following page

Count the total number of alleles at each locus and add these up to give the TOTAL ALLELES for your Test Sample and Reference Sample. Then add up the number of alleles shared by the Test Sample and the Reference Sample to give the SHARED ALLELES. Finally, use the Match Algorithm to calculate a percent match result for these two samples.

3c) Use the questions in Table 3C on the following page to help interpret your result

Comparison of many cell line samples over time has shown that there is a clear gap between related (same donor) and unrelated (different donor) samples for the vast majority of cell lines (1, 2). Related samples generally yield a result in the 80-100 % match range, and unrelated samples in the 0-55 % match range. A small number of samples may give results in the 56-79 % match range. These usually arise from a set of known variable cell lines and may need additional testing to interpret correctly – for example, using an increased number of loci or a different test method.

Sample Comparison

Table 3A: STR Profile Results

	Test Sample	***Reference Sample***
Cell line designation *e.g. name and catalog number*	**THP-1**	ATCC TIB-202
Date sample collected *(if known)*	N/A	N/A
Passage or Population Doubling *(if known)*	N/A	N/A
STR Profile Result *(enter name of kit if used)*	Promega Geneprint 10	
D5S818	11, 12	11, 12
D13S317	13, 13	13, 13
D7S820	10, 10	10, 10
D16S539	11, 12	11, 12
vWA	16, 16	16, 16
TH01	8, 9.3	8, 9.3
Amelogenin	X, Y	X, Y
TPOX	8, 11	8, 11
CSF1PO	11, 13	11, 13

Table 3B: Percent Match Calculations

TOTAL ALLELES in the Test Sample	18
TOTAL ALLELES in the Reference Sample	18
SHARED ALLELES, Test and Reference Samples	18
Percent Match for Test and Reference Samples	100%

Percent Match can be calculated using an appropriate Match Algorithm (2,3). For this Worksheet:

$$\text{Match Algorithm} = \frac{\text{SHARED ALLELES} \times 2}{\text{TOTAL ALLELES in the Test Sample} + \text{TOTAL ALLELES in the Reference Sample}}$$

Percent Match = Match Algorithm result x 100

Table 3C: Interpretation of Results

Are there multiple peaks that would be consistent with a mixture?	YES/**NO**
Is the percent match result in the range 0-55 %? This result is consistent with the two samples being unrelated (different donors)	YES/**NO**
Is the percent match result in the range 56-79 %? This result is indeterminant and may need further testing	YES/**NO**
Is the percent match result in the range 80-100 %? This result is consistent with the two samples being related (same donor)	**YES**/NO

Sample Comparison

Table 3A: STR Profile Results

	Test Sample	***Reference Sample***
Cell line designation *e.g. name and catalog number*	**NB4**	CVCL-0005 Cellosaurus
Date sample collected *(if known)*	N/A	N/A
Passage or Population Doubling *(if known)*	N/A	N/A
STR Profile Result *(enter name of kit if used)*	Promega Geneprint 10	
D5S818	13, 13	13, 13
D13S317	11, 12	11, 12
D7S820	10, 13	10, 13
D16S539	9, 9	9, 9
vWA	16, 19	16, 19
TH01	7, 9.3	7, 9.3
Amelogenin	X, X	X, X
TPOX	8, 11	8, 11
CSF1PO	11, 12	11, 12

Table 3B: Percent Match Calculations

TOTAL ALLELES in the Test Sample	18
TOTAL ALLELES in the Reference Sample	18
SHARED ALLELES, Test and Reference Samples	18
Percent Match for Test and Reference Samples	100%

Percent Match can be calculated using an appropriate Match Algorithm (2,3). For this Worksheet:

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Is the percent match result in the range 0-55 %? This result is consistent with the two samples being unrelated (different donors)	YES/**NO**
Is the percent match result in the range 56-79 %? This result is indeterminant and may need further testing	YES/**NO**
Is the percent match result in the range 80-100 %? This result is consistent with the two samples being related (same donor)	**YES**/NO

Sample Comparison

Table 3A: STR Profile Results

	Test Sample	***Reference Sample***
Cell line designation *e.g. name and catalog number*	**K562**	ATCC-CCL-243
Date sample collected *(if known)*	N/A	N/A
Passage or Population Doubling *(if known)*	N/A	N/A
STR Profile Result *(enter name of kit if used)*	Promega Geneprint 10	
D5S818	11, 12	11, 12
D13S317	8, 8	8, 8
D7S820	9, 11	9, 11
D16S539	11, 12	11, 12
vWA	16, 16	16, 16
TH01	9.3, 9.3	9.3, 9.3
Amelogenin	X, X	X, X
TPOX	8, 9	8, 9
CSF1PO	9, 10	9, 10

Table 3B: Percent Match Calculations

TOTAL ALLELES in the Test Sample	18
TOTAL ALLELES in the Reference Sample	18
SHARED ALLELES, Test and Reference Samples	18
Percent Match for Test and Reference Samples	100%

Percent Match can be calculated using an appropriate Match Algorithm (2,3). For this Worksheet:

$$\text{Match Algorithm} = \frac{\text{SHARED ALLELES} \times 2}{\text{TOTAL ALLELES in the Test Sample} + \text{TOTAL ALLELES in the Reference Sample}}$$

Percent Match = Match Algorithm result x 100

Table 3C: Interpretation of Results

Are there multiple peaks that would be consistent with a mixture?	YES/**NO**
Is the percent match result in the range 0-55 %? This result is consistent with the two samples being unrelated (different donors)	YES/**NO**
Is the percent match result in the range 56-79 %? This result is indeterminant and may need further testing	YES/**NO**
Is the percent match result in the range 80-100 %? This result is consistent with the two samples being related (same donor)	**YES**/NO

Sample Comparison

Table 3A: STR Profile Results

	Test Sample	***Reference Sample***
Cell line designation *e.g. name and catalog number*	**HL60**	ATCC CCL-240
Date sample collected *(if known)*	N/A	N/A
Passage or Population Doubling *(if known)*	N/A	N/A
STR Profile Result *(enter name of kit if used)*	Promega Geneprint 10	
D5S818	12, 12	12, 12
D13S317	8, 11	8, 11
D7S820	11, 12	11, 12
D16S539	11, 11	11, 11
vWA	16, 16	16, 16
TH01	7, 8	7, 8
Amelogenin	X, X	X, X
TPOX	8, 11	8, 11
CSF1PO	13, 14	13, 14

Table 3B: Percent Match Calculations

TOTAL ALLELES in the Test Sample	18
TOTAL ALLELES in the Reference Sample	18
SHARED ALLELES, Test and Reference Samples	18
Percent Match for Test and Reference Samples	100%

Percent Match can be calculated using an appropriate Match Algorithm (2,3). For this Worksheet:

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Table 3C: Interpretation of Results

Are there multiple peaks that would be consistent with a mixture?	YES/**NO**
Is the percent match result in the range 0-55 %? This result is consistent with the two samples being unrelated (different donors)	YES/**NO**
Is the percent match result in the range 56-79 %? This result is indeterminant and may need further testing	YES/**NO**
Is the percent match result in the range 80-100 %? This result is consistent with the two samples being related (same donor)	**YES**/NO

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