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Improving the anti-acute myeloid leukemia activity of CD123-specific engager T cells by MyD88 and CD40 co-stimulation

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SUPPLEMENTARY METHODS

Cell lines and culture conditions

Cell lines were grown in RPMI 1640 (ThermoFisher Scientific, Waltham, MA). Cells were regularly tested for mycoplasma contamination and for authenticity using the ATCC Cell line Authentication service (STR Analysis, ATCC, Manassas). HEK 293T cells were grown in IMDM (Thermo Scientific, Waltham, MA). All media was supplemented with 10-20% Fetal Bovine Serum (ThermoFisher Scientific, Waltham, MA) and 2 mmol/L Glutamax (ThermoFisher Scientific, Waltham, MA).

Generation of retroviral vectors

We generated a panel of retroviral vectors encoding CD20, CD123.ENG, and MyD88 (iM), CD40 (iC) or MyD88.CD40 (iMC), and CD20, CD19.ENG, and iMC. Briefly, cDNAs encoding a E2A self-cleaving peptide and iM, iC, or iMC were subcloned into an SFG retroviral vector encoding CD20.T2A.CD123.ENG (**Figure 1A**); iMC was also subcloned into an SFG retroviral vector encoding CD20.T2A.CD19.ENG. The sequence of all cloned constructs was confirmed by sequencing performed by Hartwell Center DNA Sequencing Core at St. Jude Children's Research Hospital with Big Dye® Terminator (v3.1) Chemistry on Applied Biosystems 3730XL DNA Analyzers (Thermo Fisher Scientific, Waltham). RD114-pseudotyped retroviral particles were generated by transient transfection in HEK 293T cells as previously described (29).

Flow cytometric analysis

For flow cytometry analysis, we utilized the following monoclonal antibodies: αCD20-APC (BD Biosciences, San Jose, CA, Clone, 2H7), αCD4-Alexa-Flour 700 (BD Biosciences, SK3), αCD8-APC-H7 (BD Biosciences, SK1), αCCR7- Pacific blue (Biolegend, San Diego, CA, G043H7), αCD45RO-PerCP-Cy5.5 (BD Biosciences, UCHL1), αPD1-BV605 (BD Biosciences, EH12.1), αTIM3-PE-Cy7 (Biolegend, F38-2E2), αCD123-APC (BD Biosciences, 7G3), αLAG3-PE (R&D Systems, FAB2319P), αCD34-PE (BD Biosciences, 581). Half a million transduced or NT T cells were washed with 1X PBS and the cells were incubated with 3µL of antibody and 0.5µL of Fixable Live Dead aqua stain (Thermo Fisher Scientific, L34965) on ice and protected from light for 30 mins. Cells were washed with 1X PBS+2% FBS. Flow cytometry was performed on a FACS Canto II or FACS LYRIC Cytometer (BD Biosciences, San Jose, CA) and the Data was analyzed on FlowJo 10.5.3 software (BD Biosciences, San Jose, CA).

Western blot

Three million transduced T cells were washed with 1X PBS and lysed in buffer containing 1X RIPA buffer and protease inhibitors (Thermo Fisher Scientific). Protein concentrations were estimated using BCA Protein Assay (Thermo Fisher Scientific) using BSA standards. Samples were denatured in Laemmli buffer (Bio-Rad, Hercules, CA) with 2-mercaptoethanol at 95°C for 5 minutes. Cell lysates (3µg/lane) were run on a 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) and transferred to nitrocellulose membrane iBlot mini (Thermo Fisher Scientific) using iBlot® 2 Gel Transfer system (23V, 4 minutes). Membranes were blocked on 1% Casein with 1X Tris buffer Saline (Bio-Rad) and then

probed with an αHA-tag-HRP (Cell signaling Technology, Danvers, MA). An anti-beta-actin (Santa Cruz Biotechnology, Dallas, TX, sc-47778) was used to detect beta-actin as a control. Blots were developed using Clarity ECL substrate (Bio-Rad) and imaged on a Li-COR Odyssey FC imaging System (Li-COR Biosciences, Lincoln, NE).

Cytotoxicity assay

Transduced or NT T cells were washed with 1X PBS and co-cultured with MOLM-13.GFP.ffluc or MV-4-11.GFP.ffluc or Kg1a.GFP.ffluc (CD123-positive) or K562.GFP.ffluc (CD123-negative) cells in a 24 well tissue culture dish (Corning) at an E:T ratio of 1:1 and incubated overnight. Cells were mixed thoroughly and 100μl of the cells were incubated with D-Luciferin. Luminescence was measured on a Tecan Infinite® 200 (Tecan, Mannedorf, Switzerland) and analyzed using Magellan Software (Tecan).

CD107a degranulation assay

MOLM13 (CD123-positive) cells were labeled with Cell Trace Violet ™ (Invitrogen, C34557) and incubated with NT T cells and CD123.ENG, CD123.ENG.iC, CD123.ENG.iM and CD123.ENG.iMC T cells at a E:T ratio of 1:2 with GolgiStop (BD Biosciences, 554724), αCD107a (LAMP-1, BD biosciences, H4A3) for five hours in the presence or absence of CID (0.5 nmol/L, B/B Homodimerizer, AP20187, Takara Bio). Cells were then washed and analyzed on FACS Lyric flow cytometer.

TCF-1 staining

Effector T cells were plated on CD123 protein coated plate overnight. Eighteen to 24 hours later, T cells were harvested and stained for TCF-1(C63D9, Cell Signaling Technology.) using eBioscience™ FoxP3 transcriptional buffer set (00-5523-00, ThermoFisher Scientific) according to manufacturer's instructions. Samples were subsequently analyzed using flow cytometry.

ELISA to determine CD123.ENG protein concentration

Non-Transduced and Transduced ENG.T cells were plated on a non-tissue culture treated plate coated with recombinant CD123 protein (1µg/mL, ab158757, Abcam, Cambridge, UK), in the presence or absence of CID (0.5 nM, B/B Homodimerizer, AP20187, Takara Bio). Co-culture supernatants were collected after 24 hours. ELISA to detect CD123.ENG protein was performed as previously described. (10).

Cytokine ELISA

$5x10^5$ CD123.ENG T cells or CD123.ENG.iC, CD123.ENG.iM or CD123.ENG.iMC T cells were plated at an E:T ratio of 1:1 with CD123-positive (MOLM-13) or CD123-negative (K562) target cells in the absence or presence of CID (0.5 nmol/L, B/B Homodimerizer, AP20187). Co-culture supernatants were collected after 24 hours and used for cytokine analysis. IFNγ and IL2 concentrations were determined using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Repeat stimulation and cytokine multiplex assay

$5x10^5$ NT, CD19-ENG.iMC, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM or CD123.ENG.iC T cells were plated at an E:T ratio of 1:1 with CD123-positive (MOLM-13.GFP.ffluc) or CD123-negative (K562.GFP.ffluc) target cells in the absence or presence of CID (0.5nmol/L, B/B Homodimerizer, AP20187). After 3 days, T cell antitumor activity was determined using a luciferase-based assay. Fresh tumor cells were added to adjust the E:T ratio to 1:1, and the stimulation was repeated until T cells no longer killed tumor cells. Culture supernatants were harvested from a coculture done in parallel, and cytokine production was measured by a custom human Cytokine/Chemokine Multiplex assay containing analytes for GM-CSF, IFNγ, IL-10, IL-13, IL-15, IL17A, IL-2, IL-4, IL-5, IL-6, IL-8, CXCL10, CCL2, CCL3, CCL, CCL5 and TNFα (EMD Millipore, Chicago, IL) as per manufacturer's instructions.

Quantitative PCR

Non-Transduced and Transduced ENG.T cells were plated on a non-tissue culture treated plate coated with recombinant CD123 protein (1µg/mL, ab158757, Abcam, Cambridge, UK), in the presence or absence of CID (0.5 nM, B/B Homodimerizer, AP20187, Takara Bio) for 12 hrs. T cells were harvested and total RNA was extracted using an automated RNA purification system (Maxwell ® RSC simplyRNA Cells Kit and RSC instrument; Promega, Madison WI). cDNA was synthesized from total RNA using SuperScript IV VILO KIT (ThermoFisher Scientific). For gene expression analysis, a panel of 14 genes with 18S RNA and GAPDH as housekeeping genes were evaluated by a TaqMan Gene expression array (ThermoFisher Scientific) using TaqMan Universal PCR

Master Mix (ThermoFisher Scientific) and Quantstudio 6 Real-Time PCR system(ThermoFisher Scientific). Gene expression was normalized to 18S RNA, and GAPDH and relative quantification were calculated with DDCT method. Primers used are listed in **Supplementary Figure 1**.

Colony forming unit (CFU) assay

NT, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM or CD123.ENG.iC T cells were co-cultured with CD34-positive bone marrow cells (Lonza, Basel, Switzerland) at an E:T ratio of 1:5 and 1:1 for 4 hours and were then plated in the presence of MethoCult (H4434, Stem Cell Technologies, Vancouver, Canada) and CID in a 6-well SmartDish® (Stemcell Technologies) and incubated for 12-14 days at 37°C. Plates were imaged using a Nikon C2 point-scanning confocal Microscope (Nikon, Tokyo, Japan) using a 4X objective. BFU-E (Burst Forming Unit – erythrocyte) and CFU-E (Colony forming unit – erythrocyte), CFU-GEMM (granulocyte, erythrocyte, monocyte and megakaryocyte) and CFU-GM (granulocyte and monocyte) were enumerated using machine learning pixel classifier with Random Forest algorithm on AIVIA 8.0 Image Analysis software (DRvision, Bellevue WA).

Primary cell coculture

Pediatric AML samples were obtained under St. Jude Children's Research Hospital IRB approved protocol, after informed consent was obtained in accordance to the Declaration of Helsinki. Three bone marrow samples of patients with active AML (M1, M5a, M4E0) were thawed and evaluated for CD123 expression using flow cytometry. AML cells were

separated from T cells by CD3 selection using MACS beads (Miltenyi Biotec, CD3 Microbeads, 130-050-101). The CD3-negative fraction containing AML cells was immediately refrozen, and CD3-positive T cells were expanded in RPMI media supplemented with 10% FBS and human IL-2 (100IU/mL Peprotech, 200-02) for 3 days. Expanded T cells were then activated with plate-bound CD3 (1µg/mL, Miltenyi Biotec, Germany) and CD28 (1µg/mL, Miltenyi Biotec, Germany) antibodies on non-tissue culture treated 24-well plates. CD19-ENG.iMC, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM or CD123.ENG.iC T cells were generated by retroviral transduction as described above. Day 10 post transduction, ENG T cells were cocultured with freshly thawed AML blasts and after 24 hours media was collected to determine IFNγ production by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

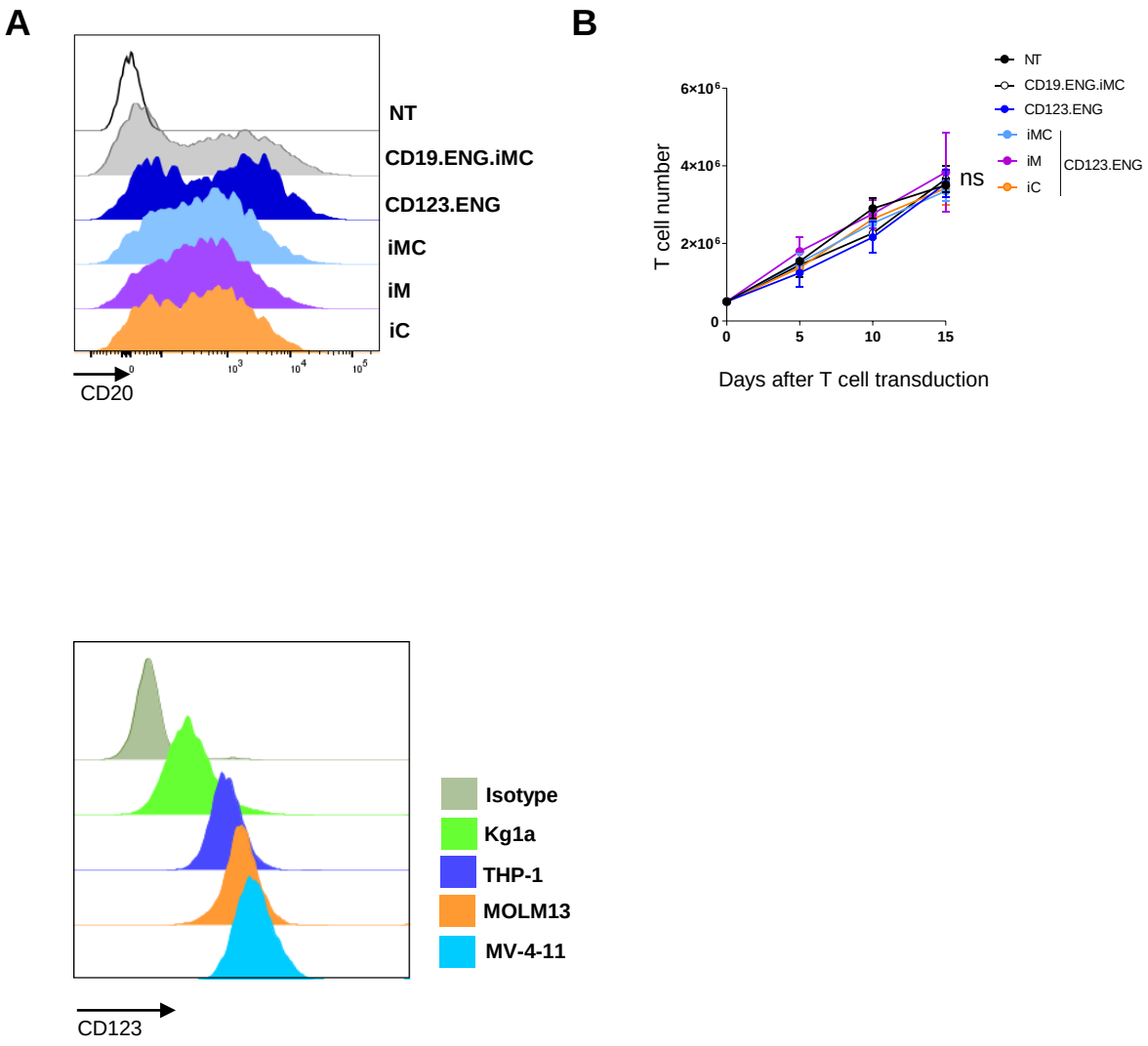
Xenograft AML models

To determine the effective dosage and efficacy of the inducible switch, 8 to 10-week old male or female NSG mice (NOD.Cg-Prkdcscid/Il2rgtm1Wjl/SzJ; St. Jude Children's Research Hospital, Memphis) were injected intravenously (iv) with $5x10^4$ MOLM-13.GFP.ffluc or $3x10^6$ THP-1.ffluc via tail vein injection. On Day 7 mice received a single iv dose of $1x10^7$ CD19-ENG.iMC, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM or CD123.ENG.iC T cells. Each mouse received 100µg of CID intraperitoneally (ip) on the day of T cell injection and then every 3-4 days for a total of 4 doses. Untreated animals served as control. AML progression was tracked by bioluminescence imaging using an IVIS-200 system (IVIS® Systems, Xenogen Corp, Alameda, CA.). For the persistence study, MOLM-13 cells were injected iv, and on day 7 animals received a single iv dose of

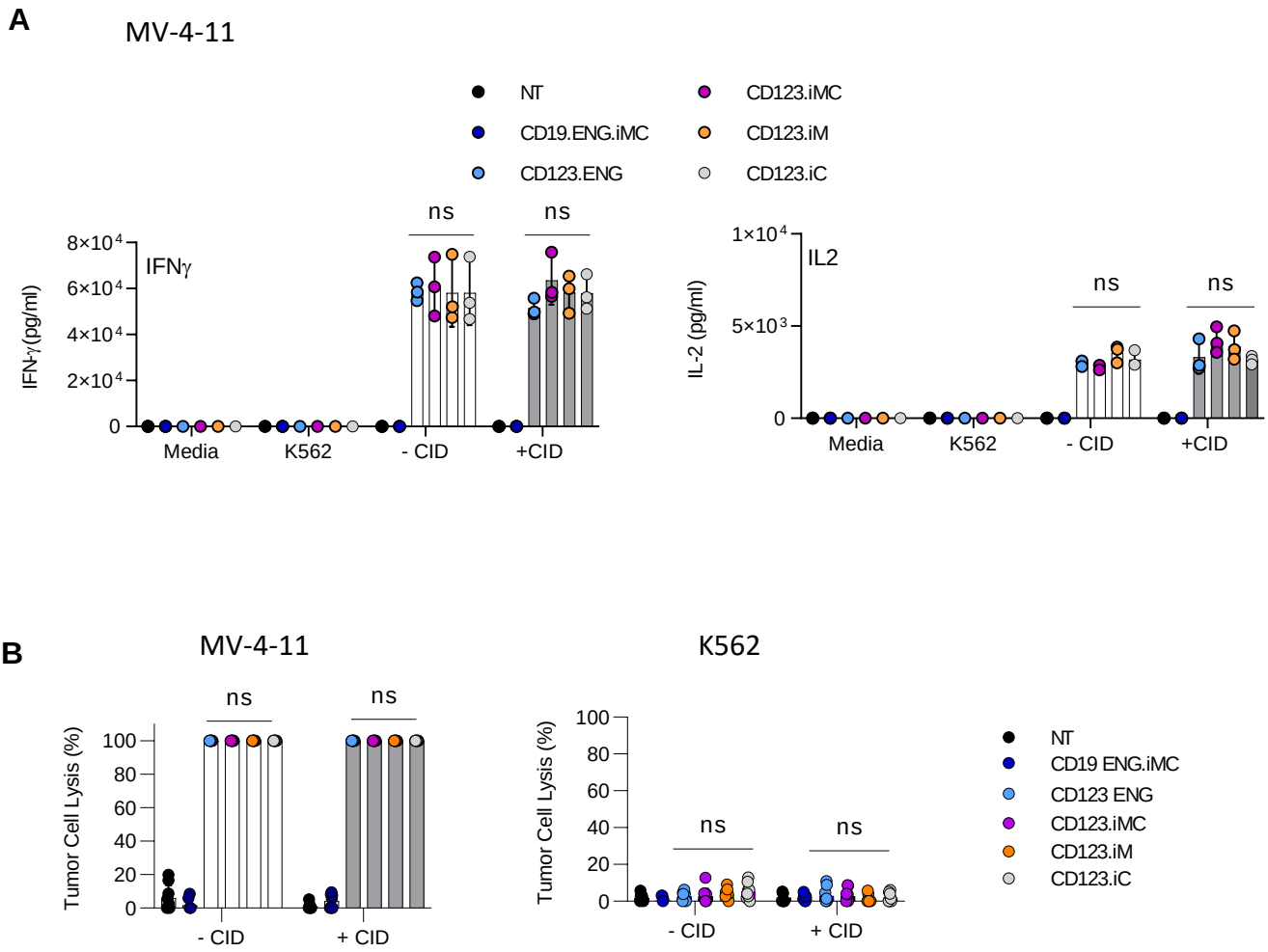
$1x10^7$ CD123.ENG, CD123.ENG.iMC, or CD123.ENG.iM T cells modified to express GFP.ffluc and received CID ip every 3-4 days for a total of 4 doses. Euthanasia was performed as prior determined time points or when animals met euthanasia criteria in accordance with St. Jude Children's Research Hospital's IACUC.

Gene	Primer Sequence
18S rRNA	CCATTGGAGGGCAAGTCTGGTGCCA
MYD88	CCCAGCATTGAGGAGGATTGCCAAA
TIGIT	CTAGAAAGAAGAAAGCCCTCAGAAT
PD1	GGCCAGGATGGTTCTTAGACTCCCC
TIM3	CTACTTACAAGGTCCTCAGAAGTGG
CXCL10	TGGCATTCAAGGAGTACCTCTCTCT
CCR5	ACATCGGAGCCCTGCCAAAAAATCA
TBET	AATGTGACCCAGATGATTGTGCTCC
BLIMP1	ACAATGATGAATCTCACACAAACAC
EOMES	ATAACATGCAGGGCAACAAAATGTA
41BB	ACCCTGGACAAACTGTTCTTTGGAT
CCL4	CGCTCTCAGCACCAATGGGCTCAGA
MYB	GCCAATTATCTCCCGAATCGAACAG
TCF7	CATCAGCCAGAAGCAAGTTCACAGG
TRAF6	AACTGAGACATCTTGAGGATCATCA
GAPDH	GGGCGCCTGGTCACCAGGGCTGCTT

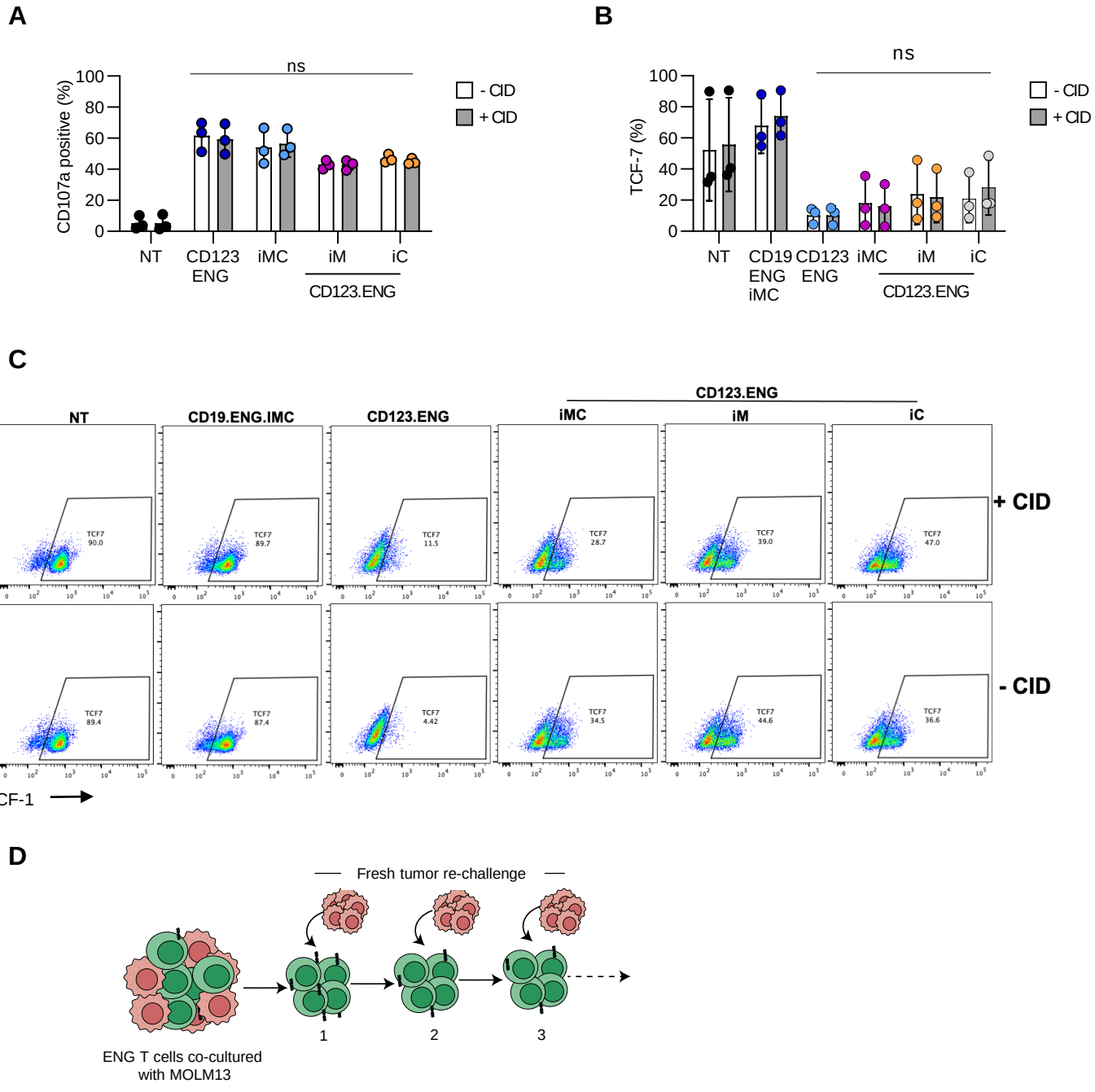
Supplementary Figure 1. Primers utilized in qPCR analysis. Table including primers utilized in qPCR analysis in **Figure 2D**.



Supplementary Figure 2: Characterization of CD123.ENG.icostim T cells and CD123+ AML cell lines. (A) Representative FACS histogram showing Non-transduced (NT), CD19.ENG.iMC, CD123.ENG, CD123.ENG.iMC, CD123.ENG.iM and CD123.ENG.iC T cells stained for CD20. **(B)** Expansion of T cells transduced with CD123.ENG and CD123.ENG.iCostim constructs compared to NT and CD19.ENG.iMC controls. (n=3 p=ns)**. (C)** Representative FACS histogram of CD123 expression (αCD123-PE, BD Biosciences, 7G3) on AML cell line MOLM-13, Kg1a, THP-1 and MV-4-11.

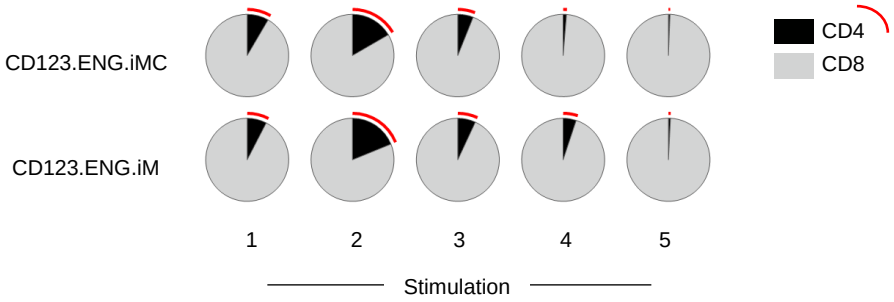


Supplementary Figure 3: CD123.ENG.icostim T cells recognize MV4-11 tumor cells (CD123+). (A) Effector cells were cocultured in the presence of media, K562 or MV-411 cells at a 1:1 E:T ratio (+/- 0.5nM CID) for 24 hours. (n=3, comparison between CD123 ENG expressing constructs: p=ns). **(B)** Luciferase-based cytotoxicity assay in which effector cells were co-cultured in the presence of luciferase tagged MV4-11 or K562 for 24 hours. (n=3, p=ns).

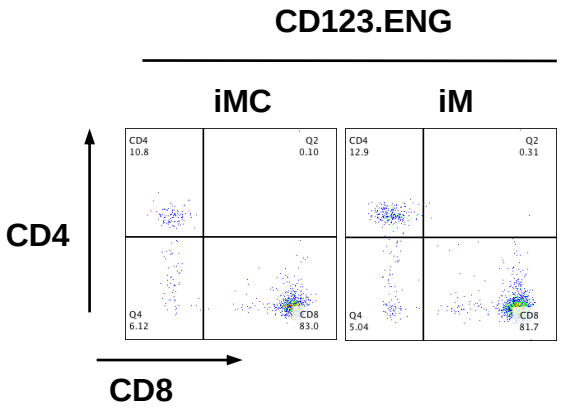


Supplementary Figure 4. Functional characterization of CD123.ENG.IMC T cells. (A) To elucidate functional differences between CD123.ENG T cell constructs, effector cells were co-cultured with MOLM-13 cells at an E:T ratio of 1:2 for 5 hours in the presence or absence of CID and determined degranulation by CD107a expression (n=3. difference between p=ns) **(B, C)** TCF-7 expression on effector cells. **(B)** summary plot (n=3 p=ns), **(C)** representative dot plots. **(D)** Experimental schema of serial stimulation assay.

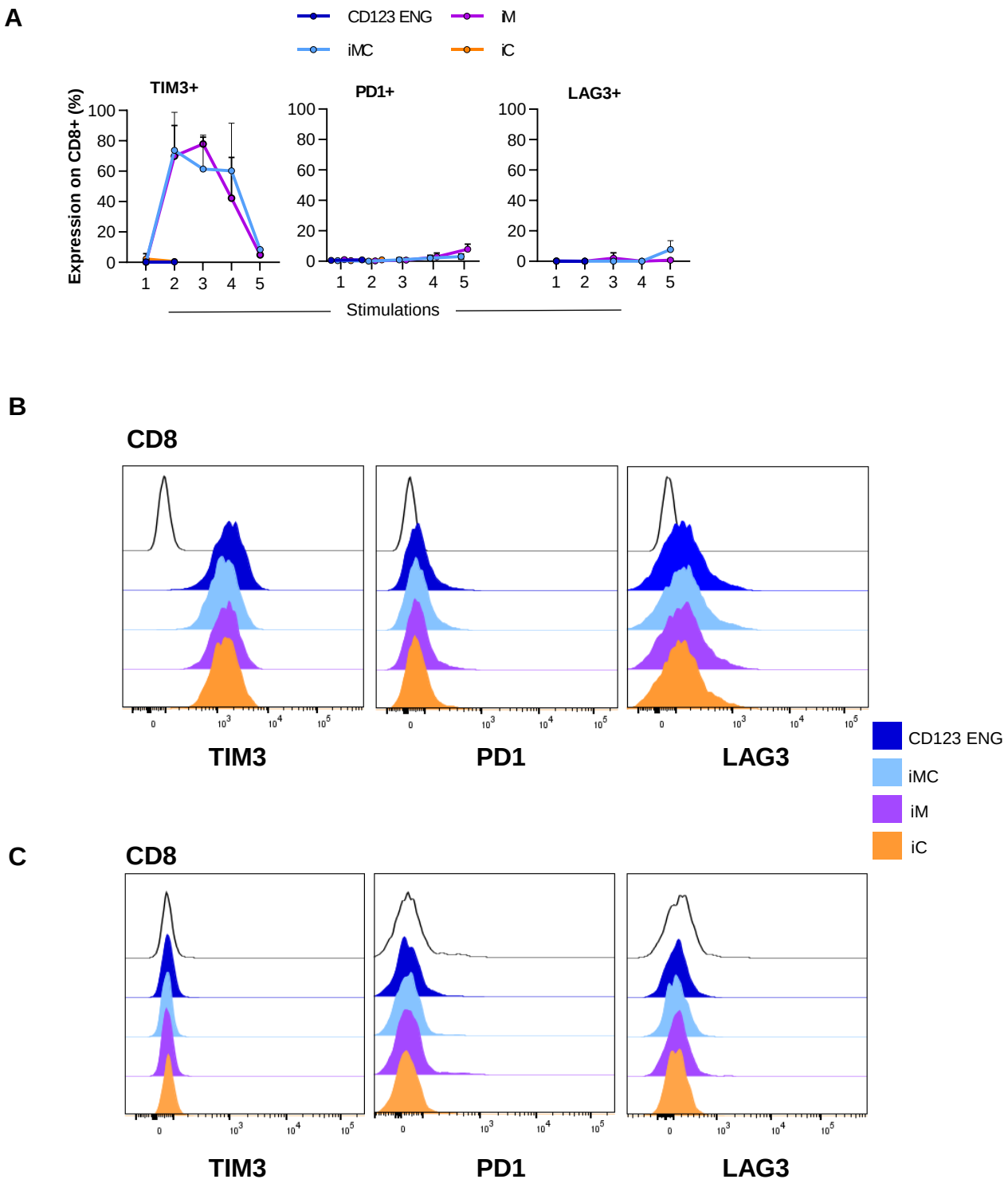
A



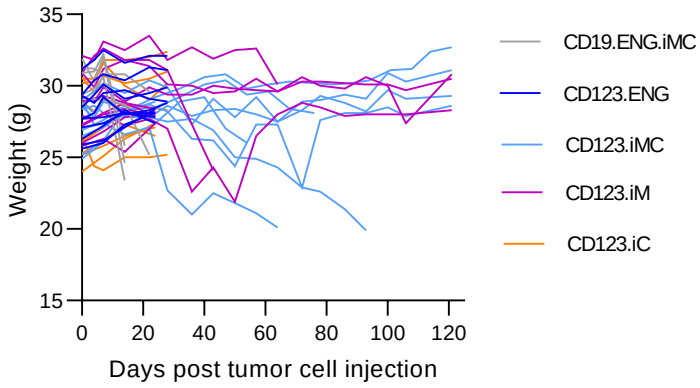
B



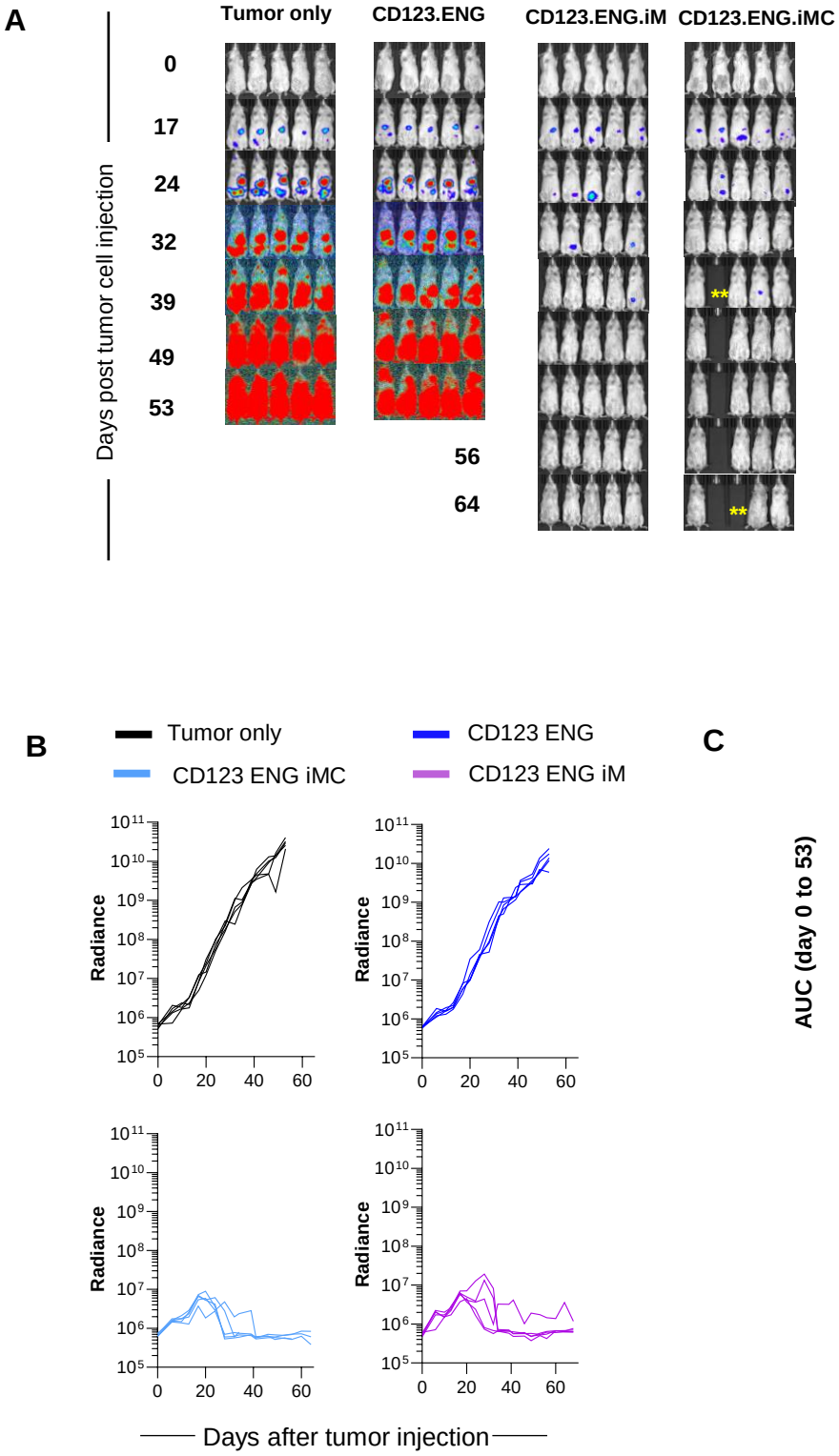
Supplementary Figure 5. Changes in CD4:CD8 Ratio in CD123.ENG.iM and CD123.ENG.iMC after multiple stimulations with CD123+ AML cells (A) Graphic representation of changes in CD4:CD8 ratios after 5 stimulations. Pie charts generated in SPICE software. (n=3) **(B)** Representative dot plots showing CD4 and CD8 staining.



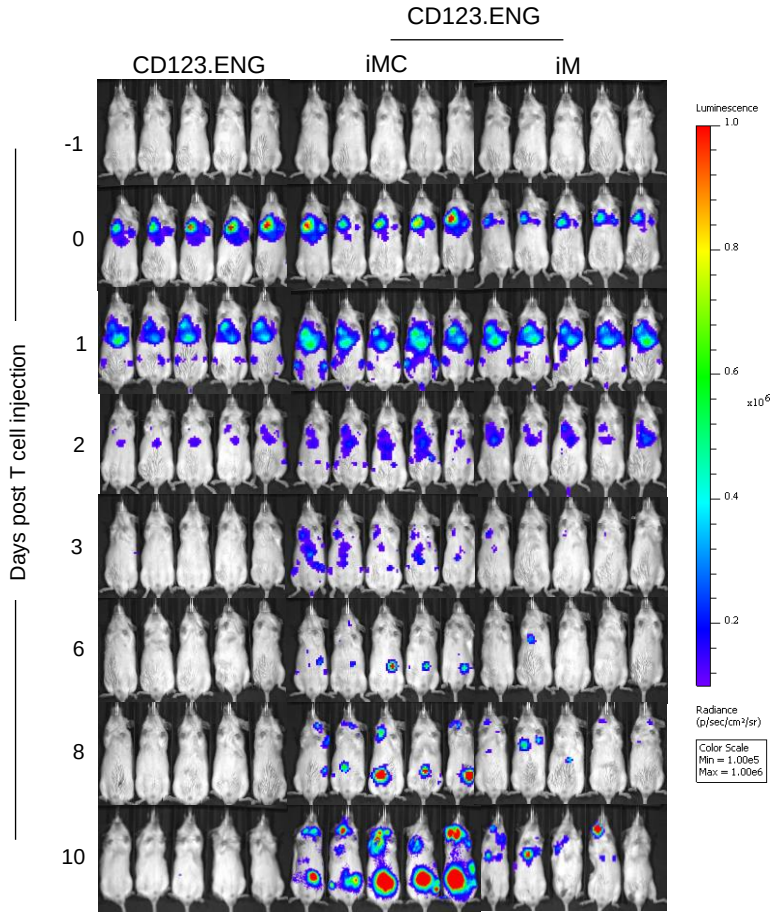
Supplementary Figure 6: Expression of TIM3, PD1 and LAG3 on CD123.ENG, CD123 ENG.iMC, CD123.ENG.iM and CD123.ENG.iC T cells. (A) Expression of TIM3, PD1 and LAG3 at baseline and subsequent stimulations **(B)** Representative histograms for TIM3, PD1 and LAG3 at baseline on CD8 T cells **(C)** Representative histograms for TIM3, PD1 and LAG3 at Stim 1 on CD8 T cells



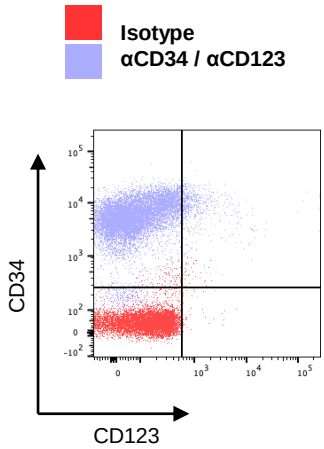
Supplementary Figure 7: Weights in grams of animals corresponding to experiments outlined in Main Figure 7.



Supplementary Figure 8: CD123.ENG.iMC and CD123.ENG.iM T cells have potent antitumor activity *in vivo* in a THP-1 AML model. THP-1.GFP.ffluc bearing mice received a single iv dose of 1x10^7 effector T cells on day 7 (n=5 animals per group), a total of 4 doses of CID were given i.p. every 3-4 days. (**A**) Experimental scheme and representative IVIS images. Stars represent animals requiring euthanasia due to non-tumor related morbidities (**= non tumor related mortality/GVHD). **(B)** Bioluminescence curves. **(C)** AUC for day 0 to day 53 comparing all groups to the tumor only control (** p<0.01, **** p<0.0001.



Supplementary Figure 9: *In vivo* expansion of CD123.ENG, CD123.ENG.iMC, and CD123.ENG.IM T cells. This is a Supplementary Figure for the *in vivo* experiment shown in Figure 8A and 8B. Representative IVIS images.



Supplementary Figure 10. Flow cytometry analysis of CD34+ cells. Representative plot for flow Cytometry analysis of hematopoietic cells (HPCs) stained for CD34 and CD123 utilized in CFU assays in Figure 7C.