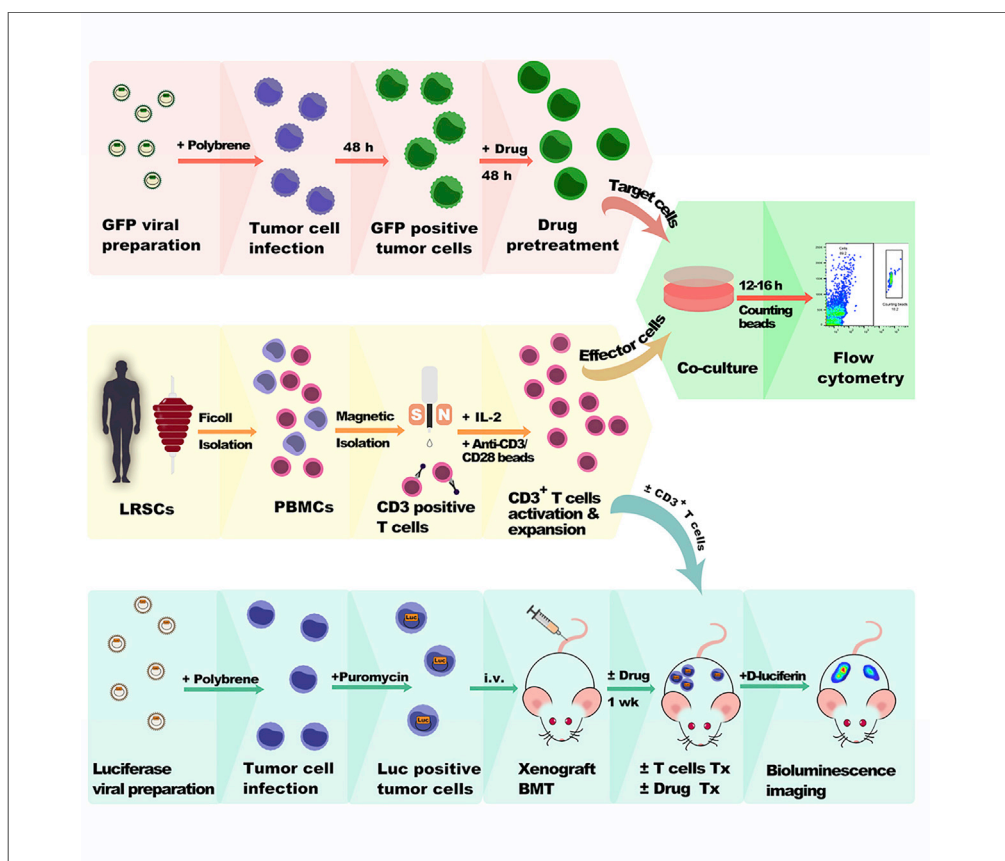


Protocol

Co-culture Systems of Drug-Treated Acute Myeloid Leukemia Cells and T Cells for *In Vitro* and *In Vivo* Study



A combination of immunotherapy and chemotherapy strategies could strengthen antitumor effects. This protocol elucidates a robust method via co-culturing drug pre-treated acute myeloid leukemia cells with $CD3^+$ T cells, derived from leukoreduction system chambers, for *in vitro* and *in vivo* study. We optimized several aspects of the procedures, including timing of drug treatment, quantification of tumor cells, and approach of combination of $CD3^+$ T cells with drug treatment *in vivo*. This enables the readouts of the interplay between drugs and T cells.

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HIGHLIGHTS

Generate $CD3^+$ T cells from leukoreduction system chambers using magnetic separation

Co-culture T cells with drug-pretreated fluorescently labeled tumor cells

Determine T cell toxicity in the co-culture system via absolute counting beads

Combine T cells and drug treatment in the xenograft mouse with luciferase-labeled AML cells

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Protocol

Co-culture Systems of Drug-Treated Acute Myeloid Leukemia Cells and T Cells for *In Vitro* and *In Vivo* StudyYangchan Li,^{1,2,3} Rui Su,^{1,3,4,*} and Jianjun Chen^{1,5,*}¹Department of Systems Biology, Beckman Research Institute of City of Hope, Monrovia, CA 91016, USA²Department of Radiation Oncology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong 510080, China³These authors contributed equally⁴Technical Contact⁵Lead Contact*Correspondence: rsu@coh.org (R.S.), jianchen@coh.org (J.C.)
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SUMMARY

A combination of immunotherapy and chemotherapy strategies could strengthen antitumor effects. This protocol elucidates a robust method via co-culturing drug pre-treated acute myeloid leukemia cells with CD3⁺ T cells, derived from leukoreduction system chambers, for *in vitro* and *in vivo* study. We optimized several aspects of the procedures, including timing of drug treatment, quantification of tumor cells, and approach of combination of CD3⁺ T cells with drug treatment *in vivo*. This enables the readouts of the interplay between drugs and T cells.

For complete details on the use and execution of this protocol, please refer to Su et al. (2020).

BEFORE YOU BEGIN

Peripheral Blood Preparation

The peripheral blood mononuclear cells (PBMCs) were enriched from leukoreduction system chambers (LRSCs) from Blood Donor Center in City of Hope (COH). The related protocol was approved by the Institutional Review Board (IRB) committee (COH Protocol # /Ref #: 18147 / 175688) and written informed consents were obtained. LRSCs were the waste products of plateletpheresis procedures. LRSCs are characterized by enrichment of mononuclear cells and depletion of most granulocytes and platelets (Boudreau et al., 2019). The frequency of PBMCs per LRSC is approximately 0.9×10^9 to 1.4×10^9 cells with more than half are CD3⁺ lymphocytes (Neron et al., 2007).

△ **CRITICAL:** Utilization of human materials must be approved by the IRB committee. Informed consent must be obtained by the donors.

Alternatives: Whole blood could be used as the source of PBMCs with a frequency of about 2×10^6 cells per mL.

Prepare NRGs Mice for Xenograft Bone Marrow Transplantation

To test the synergistic effects of a targeted therapy or chemotherapy drug plus CD3⁺ T cells treatment in a human acute myeloid leukemia (AML) cell line-derived xenograft mouse model, immunodeficient NRGs mice (RRID: IMSR_JAX:024099) were used. NRGs mice were bred and maintained in the specific-pathogen-free animal facility of City of Hope on a 12 h: 12 h light-dark cycle supplied with irradiated food and autoclaved water ad libitum.



△ **CRITICAL:** Animal procedures should be approved by the Institutional Animal Care and Use Committee and in compliance with institutional and national guidelines.

Alternatives: Other immunocompromised mice such as NSGS mice should be applicable.

Establish Stably GFP-Labeled Mono Mac 6 and Luciferase-Expression MA9.3ITD Cells

⌚ **Timing:** 1 week

Based on our previous study, we identified two small molecules, CS1 and CS2, efficiently and selectively targeting FTO. Moreover, we found that leukocyte immunoglobulin-like receptor subfamily B member 4 (LILRB4) is a direct target of FTO. FTO positively regulates LILRB4 expression in an m⁶A modification dependent manner. LILRB4 was recognized as an immune checkpoint gene due to its modulation of T cell toxicity. Via targeting FTO/m⁶A/LILRB4 axis, CS1 and CS2 treatment can increase the T cell toxicity *in vitro* and *in vivo*. AML cell lines with high expression levels of LILRB4, such as Mono Mac 6 and MA9.3 ITD are exemplified in this protocol. Mono Mac 6 is a human acute monocytic leukemia cell line derived from a 64-year old man, while MA9.3ITD derives from human CD34⁺ hematopoietic stem cells stably infected by MLL-AF9 and FLT3-ITD (Wunderlich et al., 2013). According to our preliminary data, both Mono Mac 6 and MA9.3ITD are with high endogenous expression of both *FTO* and *LILRB4* (the two genes we are focused on), can be easily infected by lentivirus, and result in leukemogenesis within 1–2 months. As to how to select the AML cell lines for study, we would like to suggest the researchers test expression of genes and/or immune checkpoint genes they are interested at first, and then evaluate whether the cells can be easily infected by lentivirus and lead to typical AML in a relatively short time (1–3 months).

△ **CRITICAL:** Before starting, make sure the HEK 293T cells are in rapid replication state and use the cells that are 75%–80% confluence.

1. On day 0, seed 1.5×10^6 HEK 293T cells per 6 cm tissue culture dish in 4 mL complete DMEM medium and incubate the cells at 37°C and 5% CO₂. The cells should reach around 50% confluence the next day.
2. On day 1, co-transfect pmiRNA1 or pLenti-CMV-Puro-LUC with pMD2.G, pMDLg/PRRE, and pRSV-Rev into HEK-293T using Effectene Transfection Reagent.
 - a. Prepare the mixture of four transfection plasmids:

Reagent	Amount per 6 cm dish	Amount per 10 cm dish
pMD2.G	0.5 µg	1.5 µg
pMDLg/PRRE	0.3 µg	0.9 µg
pRSV-Rev	0.7 µg	2.1 µg
pmiRNA1 or pLenti-CMV-Puro-LUC	1.8 µg	5.4 µg
EC Buffer	150 µL	300 µL
Enhancer	8 µL	24 µL

- b. Mix by vortexing for 1 s and incubate at 20°C for 3 min then spin down the mixture shortly.
- c. Add 25 µL (for 6 cm dish; 75 µL for 10 cm dish) Effectene Transfection Reagent to the mixture. Mix by vortexing for 10 s then incubate for 8 min.
- d. Gently aspirate medium, add 3 mL (for 6 cm dish; 9 mL for 10 cm dish) fresh DMEM complete medium to the plate.

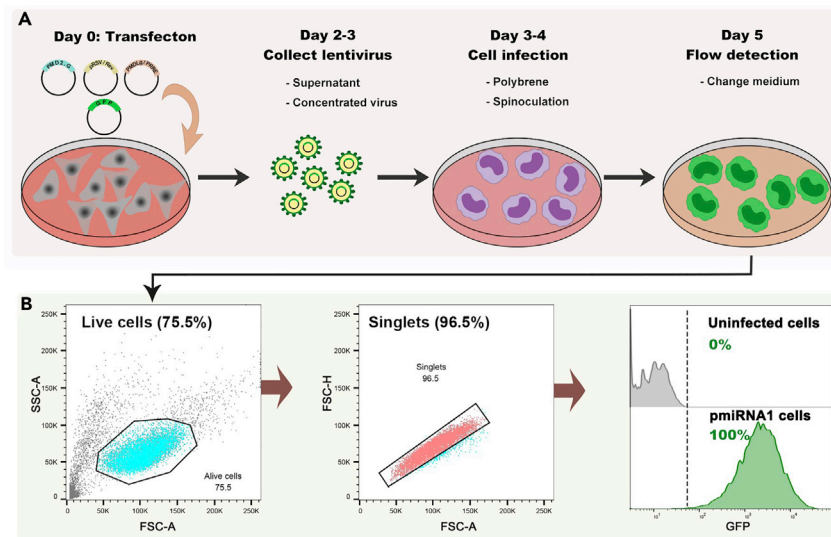


Figure 1. Generation of AML Cells Stably Expressing GFP

(A) The diagram illustrating the step-by-step method to generate stable GFP⁺ AML cells, including lentivirus packaging, lentivirus harvest, lentivirus infection, and confirmation of GFP level via cytometry flow.

(B) Detect the expression of GFP in AML cells via cytometry flow. Forward scattering area (FSC-A) and side scatter area (SSC-A) were used to initially gate cells (Gate 1). Single cells were further refined based on forward scatter height (FSC-H) and FSC-A (Gate 2) and subsequently, the GFP signal was determined (Gate 3).

- e. Add 1 mL DMEM complete medium to the tube containing the transfection mixture. Mixing by pipetting up and down several times and immediately apply the mixture dropwise onto the cells. Gently swirl the vessel to ensure even distribution.

Alternatives: Other transfection reagents such as Lipofectamine 3000, X-tremeGENE, or PEI should be applicable.

3. The following morning, carefully replace with 4 mL (for 6 cm dish; 12 mL for 10 cm dish) DMEM complete medium within 18 h.

△ CRITICAL: The supernatant should contain some viral particles. Soak all the contacted pipette tips, medium, or any other utensils in 10% bleach for at least 20 min.

4. Harvest the viral supernatant at 48 and 72 h post transfection by filtering through a 0.45 μm syringe filter.
5. Infect Mono Mac 6 cells with pmirRNA1 viral supernatant.
 - a. Seed 0.5–1.0 × 10⁶ Mono Mac 6 cells in 1 mL medium (90% RPMI 1640, 10% FBS, 2mM L-glutamine, 1 × non-essential amino acids, 1mM sodium pyruvate and 10 μg/mL human insulin) to a 6-well non-tissue culture plate.
 - b. Apply 1 mL pmirRNA1 viral supernatant and 4–8 μg/mL polybrene to the cells.
 - c. Centrifuge in a pre-warmed centrifuge at 500 × g for 90 min at 32°C.
 - d. Repeat the spinoculation using the 72-h supernatant the next day.
 - e. Check the proportion of GFP⁺ cells by flow cytometry 48 h post the second round of infection. Nearly 100% of Mono Mac 6 cells express GFP (Figure 1).

Alternatives: The tumor cells can be labeled with other fluorescent proteins such as YFP and RFP.

6. Infect MA9.3ITD cells with pLenti-CMV-Puro-LUC viral supernatant.

- a. Seed $0.5\text{--}1.0 \times 10^6$ MA9.3ITD cells in 1 mL IMDM complete medium to a 6-well non-tissue culture plate.
- b. Apply 1 mL pLenti-CMV-Puro-LUC viral supernatant and 4–8 $\mu\text{g}/\text{mL}$ polybrene to the cells.
- c. Centrifuge in a pre-warmed centrifuge at $500 \times g$ for 90 min at 32°C .
- d. Repeat the spinoculation the next day.
- e. After 48-h infection, add 2 $\mu\text{g}/\text{mL}$ puromycin to select the constitutively expressed firefly luciferase cells. The expression of luciferase can be detected upon D-Luciferin exposure as follow:
 - i. Count the MA9.3ITD-Luciferase expressed cells and uninfected MA9.3ITD cells and seed 2×10^4 cells of each in 100 μL PBS in an opaque 96-well microplate.
 - ii. Add 1 μL 10 mg/mL D-Luciferin to each well, mixed well by pipetting, and incubate at 20°C for 5 min.
 - iii. Measure the luminescence signal using Tecan INFINITE M1000 PRO or other luminometer microplate reader. The luminescence signal usually is more than 1,000 times or even much higher in the luciferase expressed cells than the uninfected cells.

Alternatives: The expression of luciferase can also be detected by PCR.

7. To ensure consistent results across different batches of experiments, one can isolate a stable Luciferase-expression clone via limiting dilution or array dilution assay. Here we take limiting dilution as an example. Prepare at least two 96-well non-tissue culture plates for selection.
 - a. Resuspend the luciferase-tagged MA9.3ITD cells in fresh complete medium, and determine cell concentration with a cell counter.
 - b. Prepare a 22 mL cell suspension at a concentration of 5 cells/mL supplemented with 2 $\mu\text{g}/\text{mL}$ puromycin. To minimize the experimental error, take out the desired number of cells in a 10-fold series dilution. For example, if the cell concentration is 8×10^5 cells/mL, then the calculated volume for 110 cells is 0.1375 μL . Firstly, mix up 137.5 μL cell suspensions (8×10^5 cells/mL) with 1237.5 μL medium to reach a cell density of 8×10^4 cells/mL. Then dilute the cells in two rounds of 10-fold dilution to reach a cell density of 8×10^2 cells/mL. Finally, mix up 137.5 μL cell suspensions (8×10^2 cells/mL) with 21.9 mL complete medium.
 - c. Apply 100 μL cell suspensions (5 cells/mL) to each well of a 96-well plate. Choose one empty well to add 100 μL about 1000 cells for focusing under the low-power field of a microscope. Incubate the cells in the incubator for two weeks.
 - d. Check and record single clones under a 400-fold microscope on day 4, 7, 10, and 14. Replenish another 100 μL fresh complete medium at the elapsed time of one week.
 - e. Transfer the expanded clones into a 24-well non-tissue culture plate. Once the single clones sufficiently expand, test the expression level of luciferase and choose the one with highly efficiently expressed luciferase and optimal growth rate.

△ CRITICAL: It is strongly advised to regularly test the cell lines for contamination with mycoplasma.

Alternatives: The viral supernatant can be concentrated using PEG-it virus precipitation solution. Aliquot and store the concentrated lentivirus at -80°C . Avoid repeated freeze-thaw cycles.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-human CD3, Brilliant Violet 785	BioLegend	Cat#317330; RRID: AB_2563507
Anti-human CD4, PE	BioLegend	Cat#357404; RRID: AB_2562036

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-human CD8, Pacific Blue	BioLegend	Cat#344718; RRID: AB_10551438
Human BD Fc Block	BioScience Inc.	Cat#564219; RRID: AB_2728082
Biological Samples		
Healthy donor LRSCs	City of Hope, blood donor center	N/A
Chemicals, Peptides, and Recombinant Proteins		
Phosphate-buffered saline (PBS), pH 7.4	Life Technologies	Cat#10010-049
0.5 M EDTA	Thermo Fisher Scientific	Cat#15575020
Ficoll Paque Plus	GE Healthcare	Cat#17-1440-02
Ammonium Chloride Solution	STEMCELL Technologies	Cat#07850
Bovine serum albumin solution, 22%	Sigma-Aldrich	Cat#A7034
CD3 MicroBeads, human	Miltenyi Biotec	Cat#130-050-101
Dynabeads Human T-Activator CD3/CD28	Thermo Fisher Scientific	Cat#11161D
Recombinant Human IL-2 Protein	R&D Systems	Cat#202-IL-010
Absolute Counting Beads	Thermo Fisher Scientific	Cat#C36950
Fetal Bovine Serum (FBS)	Gemini Bio-Products	Cat#100-106
L-Glutamine (200 mM)	Thermo Fisher Scientific	Cat#25030-081
MEM Non Essential Amino Acids Solution (100X)	Thermo Fisher Scientific	Cat#11-140-050
Sodium Pyruvate (100 mM)	Thermo Fisher Scientific	Cat#11360-070
Insulin, human recombinant, zinc solution	Thermo Fisher Scientific	Cat#12585014
Dimethyl Sulfoxide, DMSO	Fisher Bioagents	Cat#BP231-100; CAS: 67-68-5
HEPES (1M)	Thermo Fisher Scientific	Cat#15630080
IMDM	Fisher Scientific	Cat#12440061
RPMI 1640 Medium	Thermo Fisher Scientific	Cat#11875119
DMEM, High Glucose, with Pyruvate	Life Technologies	Cat#11995-073
Penicillin Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat#15-140-122
Trypsin-EDTA 0.05%	Fisher Scientific	Cat#25-300-120
Plasmocin prophylactic, 2.5 mg	InvivoGene	Cat#ant-app
Acetic Acid, Glacial (Certified ACS)	Fisher Scientific	Cat#A385500; CAS: 64-19-7
Flow Cytometry Staining Buffer	eBioscience	Cat#00-4222-26
IC Fixation Buffer	eBioscience	Cat#00-8222-49
D-Luciferin Firefly, potassium salt	Goldbio	Cat#LUCK
Polybrene	Sigma-Aldrich	Cat#H9268; CAS: 28728-55-4
Puromycin Dihydrochloride	Sigma-Aldrich	Cat#P8833; CAS: 58-58-2
Critical Commercial Assays		
Effectene Transfection Reagent	Qiagen	Cat# 301427
Experimental Models: Cell Lines		
293T	ATCC	Cat#P8833
Mono Mac 6	DSMZ	ACC-124; RRID: CVCL_1426
MA9.3ITD	Dr. James C. Mulloy (CCHMC, Cincinnati, OH)	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Mouse: NRG5	The Jackson Laboratory	RRID: IMSR_JAX:024099
Recombinant DNA		
pmiRNA1	System Biosciences	Cat#PMIRHxxxPA-1
pLenti CMV Puro LUC (w168-1)	Campeau et al., 2009	Addgene plasmid #17477
pMD2.G	A gift from Dr. Didier Trono	Addgene plasmid #12259
pMDLg/pRRE	Dull et al., 1998	Addgene plasmid #12251
pRSV-Rev	Dull et al., 1998	Addgene plasmid #12253
Software and Algorithms		
FlowJo v10	BD Life Sciences	https://www.flowjo.com/
Other		
DynaMag-2 Magnet	Thermo Fisher Scientific	Cat#12321D
33 mm syringe filter, 0.45 μm	Fisher Scientific	Cat#09-720-005
MACS MultiStand magnet	Miltenyi Biotec	Cat#130-108-933
LS Columns	Miltenyi Biotec	Cat#130-042-401
BD LSRFortessa X-20 cell analyzer	BD Biosciences	N/A
LagoX system	Spectral Instruments Imaging	N/A

MATERIALS AND EQUIPMENT

Alternatives: We used Miltenyi MACS MultiStand magnet for cell separation, while other selection magnets such as MagCelect Cell Selection Magnet from R&D Systems, EasySep magnet from STEMCELL Technologies, or DynaMag-5 Magnet from Thermo Fisher Scientific may suffice. Validation should be performed in advance if using alternative equipment.

Alternatives: While *in vivo* imaging of bioluminescence was performed with LagoX system (Spectral Instruments Imaging) and analyzed with Aura imaging software, other biophotonic imaging systems should be applicable per protocol.

Isolation Buffer for Peripheral Blood

Reagent	Final Concentration	Volume (mL)
PBS, pH 7.4	n/a	500
EDTA, 0.5 M	2 mM	2.0
Total	n/a	502

Store at 2°C–8°C for up to 24 months. Warm up to 20°C before use.

Alternatives: The EDTA can be substituted by other anticoagulants such as acid citrate dextrose (ACD), citrate phosphate dextrose, or heparin.

MACS Buffer

Reagent	Final Concentration	Volume (mL)
PBS, pH 7.4	n/a	500
EDTA, 0.5 M	2 mM	2.0
Bovine serum albumin solution, 22%	0.5%	11.6
Total	n/a	513.6

Keep the MACS buffer at 2°C–8°C for up to 6 months and degas bubbles before applying to the columns.

Alternatives: Bovine serum albumin (BSA) may be replaced with other serum products such as fetal bovine serum, human serum, and human serum albumin.

RPMI 1640 Complete Medium

Reagent	Final Concentration	Volume (mL)
RPMI 1640 medium	n/a	500
FBS (vol/vol)	10%	55.6
Penicillin Streptomycin (10,000 U/mL)	100 U/mL	5.6
Plasmocin prophylactic (2.5 mg/mL)	2.5 µg/mL	0.56
HEPES, 1 M	10 mM	5.6
Total	n/a	567.4

DMEM Complete Medium

Reagent	Final Concentration	Volume (mL)
DMEM, High Glucose, with Pyruvate	n/a	500
FBS (vol/vol)	10%	55.6
Penicillin Streptomycin (10,000 U/mL)	100 U/mL	5.6
Plasmocin prophylactic (2.5 mg/mL)	2.5 µg/mL	0.56
HEPES, 1M	10 mM	5.6
Total	n/a	567.4

IMEM Complete Medium

Reagent	Final Concentration	Volume (mL)
IMDM	n/a	500
FBS (vol/vol)	20%	125
Penicillin Streptomycin (10,000 U/mL)	100 U/mL	6.3
Plasmocin prophylactic (2.5 mg/mL)	2.5 µg/mL	0.6
Total	n/a	631.9

Acetic Acid, 100 mM

Dilute 6 µL acetic acid ($\geq 99.7\%$ wt/wt) with 994 µL sterile H₂O and filter through a 0.2 µm filter to make 1 mL 100 mM acetic acid. Store at 2°C–8°C for up to 24 months.

Recombinant Human IL-2 (rh IL-2)

The specific activity of rh IL-2 is approximately 2.1×10^4 IU/µg. Reconstitute 10 µg in 100 µL sterile 100 mM acetic acid containing 0.1% BSA to obtain a 100 µg/mL stock solution and store aliquots of 10 µL at –20°C for up to 12 months. Prepare 5×10^4 IU/mL (1000×) by mixing 2 µL 100 µg/mL IL-2 with 82 µL PBS. Avoid repeated freeze-thaw cycles.

Freezing Buffer

Mix 45 mL FBS and 5 mL DMSO to make freezing buffer. Store at 2°C–8°C for up to 6 months.

D-Luciferin, 15 mg/mL

Dissolve 1 g of D-Luciferin in 66.7 mL PBS to obtain a 15 mg/mL stock solution. Store in 1-mL aliquots at –80°C for up to 1 year.

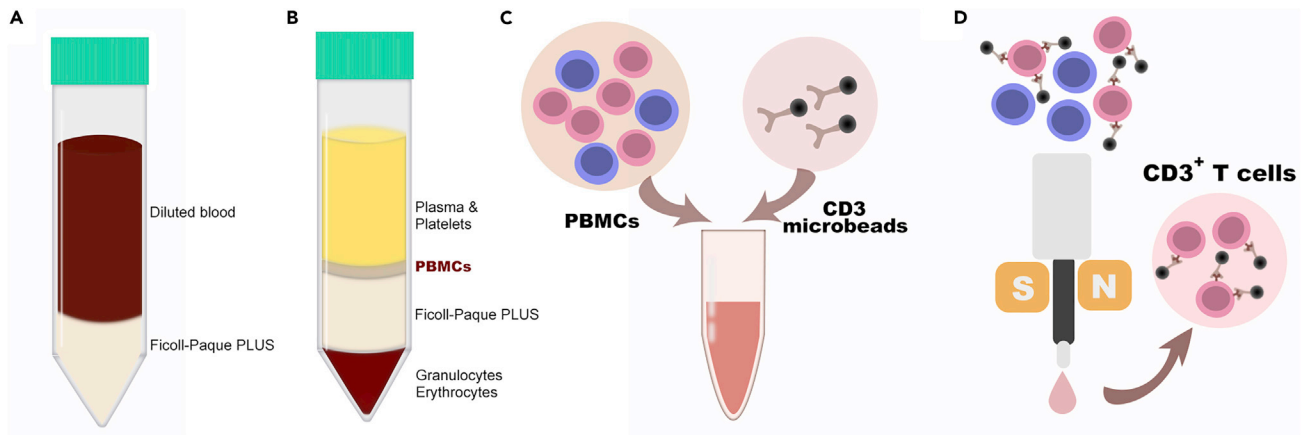


Figure 2. Isolation of CD3⁺ T Cells from LRSCs

(A) Gently layer the diluted LRSCs onto the Ficoll-Paque PLUS solution.

(B) The different layers after density gradient centrifugation. The upper layer contains the plasma/platelets; while the PBMCs are located at the interface between plasma/platelets and Ficoll-Paque PLUS buffer.

(C) Incubation of the PBMCs and CD3 magnetic microbeads.

(D) Enrichment of CD3⁺ T cells via magnetic isolation.

STEP-BY-STEP METHOD DETAILS

Isolation PBMCs from LRSCs

⌚ Timing: 2 h

LRSCs were used as sources of PBMCs in this protocol, and it can be adapted based on the different types of materials. Since aging has an impact on the reduced output of naïve T cells due to thymic involution, decreased T cell repertoire diversity, weak activation of T cells, and increased memory T cells (Salam et al., 2013). Given the circumstances that a significant decline of thymopoiesis over 40 and is less than 10% for those over 50 years of age, LRSCs from younger donors would be preferable (Hakim et al., 2005; Naylor et al., 2005). The vast majority of our healthy donors are 25–50 years old.

1. Disinfect the LRSC and tubing with 70% ethanol, and then cut each end of tubing to allow the blood flow into a 50 mL centrifuge tube.
2. Rinse the LRSC with 35 mL isolation buffer (2 mM EDTA in PBS) with a 10-mL syringe with a 22-gauge needle and mix well.
3. Add 15 mL pre-warmed 18°C–20°C Ficoll-Paque PLUS to the 50 mL centrifuge tube using a clean Pasteur pipette.
4. Carefully layer 25 mL diluted blood sample onto the Ficoll-Paque PLUS by tilting the tube (Figure 2A).

⚠ **CRITICAL:** When layering the sample do not disturb the interface between Ficoll-Paque PLUS and the diluted blood sample.

Alternatives: Other PBMC isolation tubes such as SepMate tubes from STEMCELL Technologies or Cellular Preparation Tubes from BD Biosciences could also be applicable.

5. Centrifuge at 400 × g for 30–40 min at 18°C–20°C. Troubleshooting 1

⚠ **CRITICAL:** Use centrifuge with swing buckets and with the brake-off mode to minimize the perturbation among interface. The temperature should keep at 18°C–20°C. Higher

temperatures will accelerate the sedimentation of cells leading to lower yield, while the lower temperature can taper the sedimentation of cells, causing contamination of PBMCs.

6. Aspirate the upper layer, leaving the mononuclear cell layer undisturbed at the interface (Figure 2B).
7. Carefully transfer the mononuclear cell layer to a new 50 mL conical tube.

⚠ **CRITICAL:** Remove all the interface material in a minimal volume. Transfer excessive supernatant will cause platelet contamination while removing redundant Ficoll-Paque PLUS could lead to granulocytes contamination.

8. Wash cells by bringing the volume up to 50 mL with the isolation buffer (at least three times of the volume), gently suspend the cells by drawing in and out of a Pasteur pipette, and centrifuge at $300 \times g$ for 10 min at 18°C – 20°C . Carefully remove the supernatant completely.
9. Resuspend the cell pellet in 50 mL isolation buffer, mix 10 μL cell suspension and 10 μL trypan blue staining, apply 10 μL mixture to an automatic cell counter and calculate the cell concentration and viability.
10. Pellet the cells at $200 \times g$ for 10 min at 18°C – 20°C . Aspirate supernatant completely and then proceed to $\text{CD}3^{+}$ T cells isolation.

Note: For one LRSC, the number of PBMCs by this step should be 0.5×10^9 – 2×10^9 , half of which are $\text{CD}3^{+}$ T cells. Determine the cell number of PBMCs used for the isolation and the rest could be cryopreserved.

Optional: Add 15 mL ammonium chloride to lyse the red blood cells and incubate for 5 min at 20°C if the cell pellet is red. Then wash with the isolation buffer.

Optional: Resuspend the cell pellet in freezing buffer to achieve a cell density of 10 – 50×10^6 cells per mL, and add 1 mL freezing mix to each cryovial. Immediately place the cryovials into a freezing container and store at -80°C . For long-term storage, transfer the cryovials to the liquid nitrogen the next day.

⏸ **Pause Point:** The PBMCs can be stored at -80°C for up to one month or liquid nitrogen for later separation.

Isolate $\text{CD}3^{+}$ T Cells Using Magnetic Beads Technique

⌚ **Timing:** 2 h

To acquire optimal purity and cell viability, work fast, keep cell cold, and use pre-cool solutions. Pre-cool the centrifuge to 4°C . If working with cryopreserved PBMCs, pass the cells through a 30 μm mesh cell strainer to remove cell clumps. Alternatively, the thawed cell pellet can be incubated for 15 min at 37°C in 5 mL of RPMI 1640 complete medium with 20–200 U/mL DNase I to prevent unwanted cell clump.

11. Resuspend the cell pellet in 80 μL of MACS buffer per 10^7 total cells in a 15 mL or 50 mL centrifuge tube.

Note: For larger cell amount, scale up the volume. For example, resuspend 2×10^7 cells in 160 μL MACS buffer.

12. Add 20 μL of $\text{CD}3$ MicroBeads per 10^7 total cells. Mix well and incubate for 15 min in the refrigerator (2 – 8°C). Flick the bottom of the tube to mix up every 5 min (Figure 2C).

13. Wash cells by adding 1–2 mL of MACS buffer per 10^7 cells, mix by drawing in and out of a pipette, and centrifuge at $300 \times g$ for 10 min at 4°C . Discard the supernatant completely.
14. Resuspend the cell pellet gently in 500 μL of MACS buffer per 10^8 cells, avoid air bubbles during pipetting.
15. Place LS column in the magnetic field of MACS Separator. Pre-wet the column with 3 mL MACS buffer.
16. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells ([Figure 2D](#)).
17. Wash column with 3 mL of MACS buffer for three times.

△ CRITICAL: Work gently, apply buffer with bubbles onto the column may clog the column. If bubbles appear on the surface, remove it with a pipette or a vacuum filter.

18. Remove the column from the separator and place it on a 15 mL centrifuge tube.
19. Pipette 5 mL MACS buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column. Repeat it with another 5 mL MACS buffer.
20. Determine the cell number and centrifuge at $300 \times g$ for 10 min at 4°C . **Troubleshooting 2**

Note: More than half of the cells in PBMCs from LRSCs are CD3^+ T cells. Hence, when working with 20×10^6 PBMCs, you will harvest around $5 - 10 \times 10^6$ T cells.

Note: Take out the desired number of CD3^+ T cells for downstream assays, and the rest of CD3^+ T cells could be cryopreserved.

Alternatives: Negative selection methods (such as the Dynabeads untouched human T cells kit from Thermo Fisher Scientific) can be used to enrich the untouched T cells, which will eliminate the potential risk to activate T cell receptor (TCR). While, in our present study, the T cells isolated by positive selection with CD3 microbeads will be activated immediately after isolation. Thus, the positive selection has little effect on this study to test T cell cytotoxicity.

21. Perform flow cytometry to determine the proportion of CD3^+ , CD4^+ , and CD8^+ T cells (Optional).
 - a. Pellet 2×10^6 T cells by centrifuge at $300 \times g$ for 5 min at 4°C .
 - b. Resuspend T cells in 500 μL ice-cold flow cytometry staining buffer with 5 μL human Fc blocker (1:100) (BioScience Inc Cat#564219, RRID: AB_2728082), and then aliquot into five 1.5 mL EP tubes: one as unstained control, three as single staining, and the rest one as combined staining.
 - c. To the four tubes, add 1 μL anti-CD3-BV785 (BioLegend Cat#317330; RRID: AB_2563507), 1 μL anti-CD4-PE (BioLegend Cat#357404, RRID: AB_2562036), 1 μL anti-CD8-Pacific Blue (BioLegend Cat#344718, RRID: AB_10551438), and all of these three antibodies combo in sequence.
 - d. Mix well by pipetting up and down, and then incubate for 30 min at 4°C in the dark. Mix well every 10 min.
 - e. Wash the cells twice with 1 mL PBS and centrifuge at $300 \times g$ for 5 min at 4°C .
 - f. Add 100 μL IC Fixation Buffer (eBioscience Cat# 00-8222-49) to cells in 100 μL flow staining buffer, gently agitate and store up to 3 days in 2°C – 8°C in the dark.

Alternatives: 2%–4% paraformaldehyde in PBS can be used to fix the cells.

- g. Transfer the cells to the flow tube with 300 μL PBS and record on a suitable flow cytometer. Examples of flow cytometry plots are shown in [Figure 3](#).

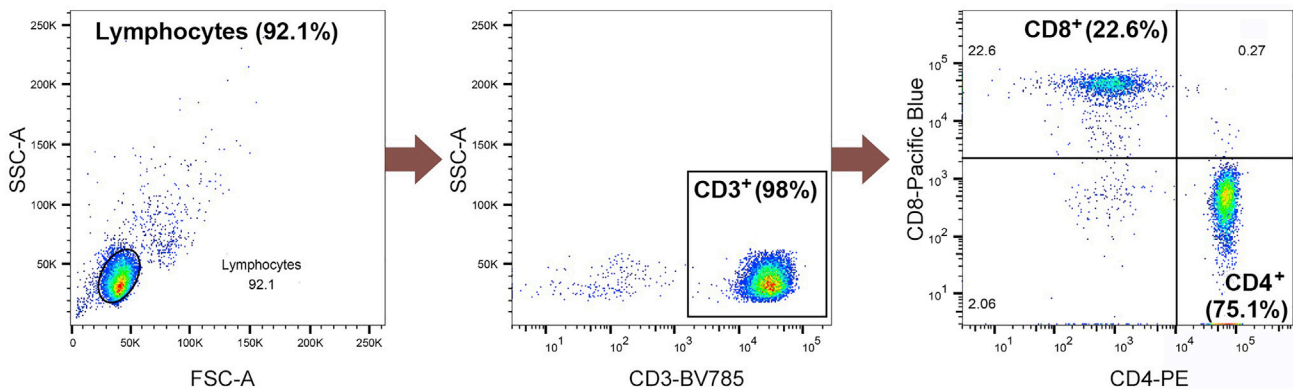


Figure 3. Representative Flow Cytometry Plots Showing the Percentage of CD3⁺, CD4⁺, and CD8⁺ T Cells Isolated from LRSCs Using the Magnetic Technique

⏸ Pause Point: The CD3⁺ T cells can be stored at -80°C up to one month or liquid nitrogen for long-term storage.

Note: When executing the cryopreservation of CD3⁺ cells at a cooling rate of $1^{\circ}\text{C min}^{-1}$ and thawing the cells rapidly (<1 min) in a 37°C water bath, the cell viability ranged from 80%–90%, and for those survived from the freeze-thaw process, the proliferation rate was unaffected (Baboo et al., 2019).

⚠ CRITICAL: Most of the T cell subpopulations showed little difference in numbers during cryopreservation, however, the percentage of activated CD8⁺ CD38⁺ HLADR⁺ cell subtype decreased during cryopreservation (Weinberg et al., 2009). Considering the variation between fresh and frozen T cells, it is highly recommended to use fresh T cells for studies.

CD3⁺ T Cells Culture and Activation

⌚ Timing: 1–2 weeks

22. Prepare anti-CD3/CD28 Dynabeads for T cell activation, which mimic *in vivo* T cell activation from antigen-presenting cells. Wash Dynabeads before adding to the T cell culture medium. The concentration of beads is 4×10^7 beads/mL, and the ratio of beads to cells is 1:1.
 - a. Resuspend the Dynabeads in the vial by vortex for more than 30 s, or in a tilt and rotate manner for 5 min.
 - b. Transfer the desired volume of Dynabeads (bead-to-cell ratio of 1:1) to a 1.5 mL EP tube and add at least 1 mL MACS buffer and mix well.
 - c. Place the tube on a DynaMag-2 magnet for 1 min and discard the supernatant.
 - d. Remove the tube from the magnet and resuspend the washed Dynabeads in the same volume of culture medium as the initial volume of Dynabeads taken from the vial.

⚠ CRITICAL: Never use a less or excess recommended volume of Dynabeads, which may lead to insufficient activation or inhibition of expansion.

23. Resuspend the cells at a density of $1\text{--}1.5 \times 10^6$ cells/mL in RPMI 1640 complete medium supplemented with 50 U/mL IL-2, and anti-CD3/CD28 dynabeads in a 6-well or 12-well non-treated culture plate. Incubate the cells at 37°C and 5% CO_2 . Upon activation for 2 to 3 days, CD3⁺ T cells could strongly bind to the dynabeads and are ready for downstream applications.

Alternatives: Other culture media, such as 45% RPMI 1640 and 45% Click's Medium supplemented with 10% FBS, OpTmizer T Cell Expansion SFM, or other equivalent media could be used for T cell culture.

Note: The T cells can be also activated by anti-CD3/CD28/CD137-coated beads. For activation and expansion of CD8⁺ T cells, add anti-CD3/CD28/CD137-coated beads, 10 ng/mL recombinant human IL-7, and 5 ng/mL recombinant human IL-15 for the homeostatic proliferation of CD8⁺ T cells and stimulation of memory CD8⁺ T cells (Wallace et al., 2006). We did not observe a significant difference of the CD3⁺ T cells generated by anti-CD3/CD28 or anti-CD3/CD28/CD137-coated beads.

24. Check the cells daily and count the cells at least twice a week. When the medium turns yellow or the cell density exceeds 2.5×10^6 cells/mL, split the cells to a density of $0.5\text{--}1.0 \times 10^6$ cells/mL in culture medium supplemented with 50 U/mL IL-2.
25. When the cells manifest signs of exhaustion, typically shrinkage of cell size, irregular elongated shape, and proliferation arrest, usually after 7–10 days of expansion, restimulate T cells with additional anti-CD3/CD28 Dynabeads. [Troubleshooting 3](#)
 - a. Thoroughly pipette the cell-beads mixture, then transfer the cells into a sterile tube.
 - b. Place the tube onto the magnet at least for 1 min.
 - c. Collect the supernatant to a new tube and count the cell number.
 - d. Pellet the cells by centrifuge at $300 \times g$ for 5 min at 20°C.
 - e. Calculate the desired amount of Dynabeads, and repeat step 22.
 - f. Seed the cells at a density of $0.5\text{--}1.0 \times 10^6$ cells/mL and replenish 50 U/mL IL-2.
 - g. Check the cells daily, and subculture the cells when the medium turns yellow or the cell density exceeds 2.5×10^6 cells/mL.

Note: The T cells remain cytotoxic after restimulation. However, it should be noticed that repeated stimulation will upregulate the inhibitory factors, such as PD-1, LAG-3, TIM-3, CTLA-4, and the transcription factor TOX (Dunsford et al., 2020). According to our experience, two rounds of stimulation with anti-CD3/CD28 have little effects on cytotoxic efficiency.

Co-culture CD3⁺ T Cells with Drug Pre-treated Tumor Cells

⌚ **Timing:** 1 day

This protocol aims to explore the combined effect of drug treatment and immunity. Under the circumstances that CD4⁺ T cells have been demonstrated to potentiate the function of CD8⁺ T cells, hence total CD3⁺ T cells are used to study the combined effects. Researchers can isolate CD8⁺ cells, activate them with anti-CD3/CD28 or CD3/CD28/CD137 dynabeads, and co-culture them with leukemia cells. Here, we label the tumor cells with GFP, then treat the cells with desired drug concentration for 48 h, and co-culture the drug pre-treated tumor cells with activated CD3⁺ T cells.

26. Seed Mono Mac 6-GFP cells into a 6-well plate with 5×10^5 cells /well in 4 mL culture medium. Add the drugs and vehicle control accordingly, and incubate for 48 h.

Note: The cell number, drug concentration, and incubation time should be optimized before co-culture. Here we used 0.1% DMSO, 30 nM CS1, 100 nM CS2 and 300 nM CS2.

27. After 48 h, harvest the cells and centrifuge at $300 \times g$ for 5 min and then resuspend in 1–2 mL culture medium.
28. Count the cell numbers and adjust the cell concentration as 4×10^5 cells per mL.
29. Seed 100 μ L Mono Mac 6-GFP cells per well into a 48-well plate \times 9 wells for each group.

Note: A 96-well plate should be applicable.

30. Determine the effector-to-target (E: T) ratio. Here we used E: T ratios of 1:1 and 2:1. Adjust the T cells concentration as 4×10^5 cells/mL and 8×10^5 cells/mL and supplement with 100 U/mL IL2 (2×).

Note: The effector-to-target ratios can be ranged from 1:2 to 50:1 or even higher. When working with higher E: T ratio (above 10:1), shorter co-culture time (4–6 h) is preferred, while working with relative lower E: T ratio (below 10:1), 12–18 h to 72 h co-culture period would be desirable (Deng et al., 2018; Andersch et al., 2019; Nelson et al., 2019; Neubert et al., 2016). In consideration of the varied magnitude of T cell-mediated cytotoxicity among different cell types, a pretest of co-culture duration is recommended.

31. Mix the tumor cells in step 29 with 100 μ L CD3⁺ T cells from step 30 in triplicate wells accordingly. For the untreated well, add 100 μ L culture medium with 50 U/mL IL2. Incubate at 37°C for 12–18 h.
32. The next day morning, transfer cells to the flow tube, wash each well with 200 μ L PBS and transfer it to the corresponding tube.
33. Count tumor cells by flow cytometry using CountBright absolute counting beads. **Troubleshooting 4**
- Completely resuspend the CountBright absolute counting beads suspension by gentle vortexing for 30 s.
 - Immediately after vortexing, apply 50 μ L bead suspensions (50,000 beads in 50 μ L) to each flow tube.
 - Briefly centrifuge to bring all of the leftovers on the lateral walls to the bottom of tubes.
 - Vortex well and then run on the flow cytometer.
 - Record at least 1000 beads event. An example of gating method is shown in [Figure 4](#).

△ CRITICAL: It is imperative to avoid cell loss at this step. Please **DO NOT** centrifuge the cells and wash them with PBS, which will lead to unexpected cell loss.

△ CRITICAL: Researchers should also confirm that the counting beads are mixed very well. The number of beads added into the flow tube will directly determine the number of leukemia cells.

Note: A less volume of counting beads should also work. To minimize the error, dilute the beads with PBS in advance and then apply 100 μ L diluted beads to each sample.

34. Calculate the number of tumor cells in each sample using the following formula:

$$\text{Total number of tumor cells} = \frac{\text{Events of GFP}^+ \text{ cells}}{\text{Events of beads}} \times \text{Number of beads}$$

The percentage of live GFP⁺ cells in the effectors-added group relative to the untreated group is subtracted from 100 and denoted as percent cytotoxicity.

$$\begin{aligned} \text{Percent Killing\%} &= \left(1 - \frac{\text{Total number of tumor cells in the T cells-added group}}{\text{Total number of tumor cells in the untreated group}} \right) \times 100\% \\ &= \left(1 - \frac{[\text{Events of GFP}^+ \text{ cells} / \text{Events of beads in the T cells – added group}]}{[\text{Events of GFP}^+ \text{ cells} / \text{Events of beads in the untreated group}]} \right) \times 100\% \end{aligned}$$

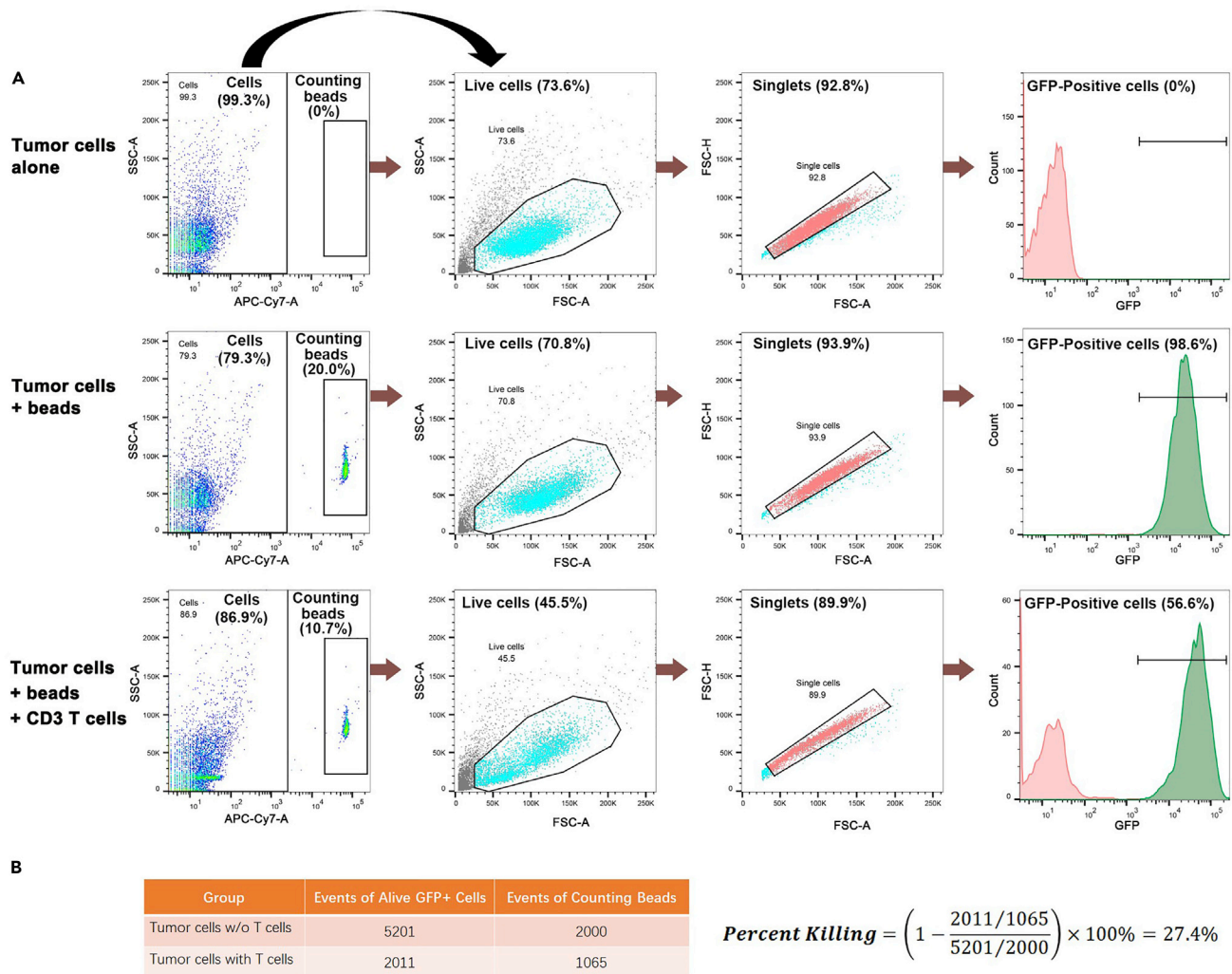


Figure 4. Representative Data Showing How to Calculate T Cell Toxicity

(A) Gating strategy of tumor cells and counting beads for the co-culture assay. The APC-Cy7-A was used to distinguish cells and counting beads. (B) Representative data showing the calculation of killing percentage based on the counting beads method.

Combination of Drugs and CD3⁺ T cells *In Vivo* via Xenograft Model Transplanted with MA9.3ITD AML Cells

⌚ Timing: 1–2 months

The AML Xenograft model subjected to FTO inhibitors and T Cells treatment is employed to access the effects of targeting the FTO/*m⁶A*/*LILRB4* axis on AML progression and immune invasion *in vivo*. Tumor progression is monitored and envisioned by *in vivo* bioluminescence imaging (BLI). MA9.3ITD cell lines are exemplified in this protocol, other AML cell lines with high endogenous FTO and *LILRB4*, such as Mono Mac 6, THP1, and MV4-11, also should be applicable. Given the immunocompromised microenvironment, it is not necessary to irradiate the NRGs mice before cell injection.

35. Injection of luciferase-tagged MA9.3ITD cells into NRGs mice
 - a. Harvest luciferase-tagged MA9.3ITD cells and pellet the cells by centrifuge at 300 × g for 5 min at 4°C.

- b. Resuspend the pellet in 5 mL ice-cold PBS and count the cell number.
- c. Pellet the cells by centrifuge at $300 \times g$ for 5 min at 4°C .
- d. Resuspend the pellet in PBS and adjust the cell concentration as 5×10^5 cells/mL. Keep the cells on the ice.
- e. Raise the mouse's body temperature with an incandescent lamp to induce peripheral vasodilatation.
- f. Place the NRGs mouse in a restrainer, rotate the tail to position the vein on top, and disinfect the tail with isopropanol.
- g. Inject 200 μL cell suspensions (1×10^5 cells) per mouse into the vein using a 27–28 gauge needle size and a 1/2-mL syringe.

Alternatives: A mouse tail illuminator restrainer can be used, which efficiently warms a mouse's tail and minimize whole-body heat stress to a great extent.

36. One week post BMT, randomly allocate the mice into six groups, PBS, T cell, CS1, CS1 plus T cell, CS2, and CS2 plus T cell group.
37. Prepare activated CD3^+ T cells for injection.
 - a. After activation CD3^+ T cells using anti- $\text{CD3}/\text{CD28}$ Dynabeads for 2–3 days, harvest the cells and centrifuge at $300 \times g$ for 5 min at 4°C .
 - b. Discard the supernatant, and resuspend the pellet in 5 mL ice-cold PBS, thoroughly pipette the cells/beads suspension, and then place the suspension onto a magnet separator until the beads have collected at the side of the tube (approximately 1 min).
 - c. Transfer the supernatant to a new 15 mL centrifuge tube.
 - d. Wash the beads twice with PBS and each time thoroughly pipetting to increase cell recovery, capture the beads using the magnetic separator and transfer the supernatant to the centrifuge tube.
 - e. Determine the cell numbers using an automatic cell counter.
 - f. Pellet the cells by centrifuge at $300 \times g$ for 5 min at 4°C .
 - g. Resuspend the CD3^+ T cells in ice-cold PBS and adjust the cell concentration as 25×10^6 cells/mL. Keep the cells on the ice.
38. For the T cell treatment groups, each mouse receive 5×10^6 activated CD3^+ T cells, which are suspended in 200 μL and administrated through tail vein injection on day 7 and 14 post BMT.

Note: The injection amount of T cells and time may be varied in different xenograft models. The total amount of injected T cells can be up to 20×10^6 cells and can be injected weekly. To track the localization, T cells can be tagged with renilla luciferase and detected with benzyl coelenterazine as substrate (Vilalta et al., 2009).

Alternatives: Retro-orbital injection can be used instead of tail vein injection.

39. Begin drug treatment one week after BMT, and each mouse is administrated every other day with PBS, 5 mg/kg/day CS1 or CS2 for 5 times in total.

Note: The start time, dosage, and times of drug treatment should be optimized per xenograft mouse model.

40. To envision the leukemic progression, perform BLI weekly. **Troubleshooting 5**
 - a. Thaw 15 mg/mL D-Luciferin stock solution at 20°C .
 - b. Weigh the mice, and inject intraperitoneally with 150 mg/kg D-Luciferin.

Note: Inject 10 μL of Luciferin stock solution per gram of body weight. For example, inject 200 μL for 20 g body weight.

Note: The injection site is located at the right low quadrant, the intersected point of the horizontal line drawn joining the upper border of two knees and the vertical paramedian line, which is 5–10 mm lateral to the midline.

c. Set a timer for 10 min.

△ **CRITICAL:** Researchers should make sure that the time between D-Luciferin injection and luminescence image is equal for each mouse from each group. Otherwise, the luminescence signals are not comparable.

Note: A kinetic study of luciferase should be performed in advance to evaluate the time of peak luciferase signal and plateau phase for different animal models. Usually, it takes 10–20 min to reach the peak signal after injection.

d. Anesthetize the mice using isoflurane and place the sedated mice in a supine position into a black image chamber equipped with an anesthesia system.

e. After 10-min injection, transfer the imaging chamber into the Lago X and capture the luminescence signal using the Aura imaging software with a consistent exposure time setting.

41. Transfer the mice to the previous cages and wait for anesthesia recovery.

42. Mice were monitor closely and euthanized once they succumbed to signs of distress such as poor grooming, hunched posture, and hind-limb paralysis.

EXPECTED OUTCOMES

This protocol describes an efficient method to generate the CD3⁺ T cells from the PBMCs using by-products of LRSCs. Using the magnetic separation method, we observed a high enrichment of CD3⁺ T cells (Figure 3). By supplementing the anti-CD3/CD28 Dynabeads and IL-2, we observed tens of hundreds of folds of expansion of CD3⁺ T cells *in vitro*.

The co-culture assay provides a feasible and sensitive method to co-culture T cells with GFP-labeled suspension cells and enumerate tumor cells and determine T cell toxicity via flow cytometry with absolute counting beads. It is highly adaptable to evaluate the effects of drug combinations and interrogate the tumor intrinsic mechanisms that influence the susceptibility of immune response.

The combination of T cells and novel drug treatment *in vivo* suggested the synergistic effect of immunotherapy and chemotherapy. The utilization of BLI envisions the tumor progress and is sufficiently sensitive to be a pharmacodynamic readout of the efficacy.

LIMITATIONS

This co-culture protocol is mainly applicable to a panel of suspension cell lines, and it needs to be optimized when working with adherent cell lines.

It is worth noted that the positive isolation of CD3⁺ T cells may prime and activate T cells via antibody-CD3 interaction (Kamala, 2008). Therefore, for the studies requiring untouched naïve CD3⁺ T cells, negative isolation with different antibody cocktails should be used.

The activation of T cells in this protocol is immensely based on repeated stimulation of anti-CD3/CD28 Dynabeads independent of major histocompatibility complex (MHC) restricted mechanisms (Dunsford et al., 2020; Nelson et al., 2019). Nevertheless, there are still some specific antitumor effects contributed by the gamma delta ($\gamma\delta$) T cells and natural killer T cells, which account for 3%–14% and 0.05%–0.12% in the PBMCs, respectively (Bernin et al., 2016; Gomes et al., 2010). Unlike conventional cytotoxic lymphocytes kill tumors in an MHC-restricted manner, $\gamma\delta$ T cells exert tumoricidal effects in a non-MHC manner (Pluhar et al., 2015). As for NKT cells, upon activation by the CD3/CD28

beads, they can promote lysis of target cells in a CD1d-dependent manner (Iyoda et al., 2010; Jahnke et al., 2019). Therefore, to generate the classical neoantigen reactive cytotoxic T lymphocytes, the prerequisite is that the T cells are derived from autologous, either tumor-infiltrating lymphocytes or from PBMCs, or share the same HLA allele with tumor cells like HLA-A*02:01 allele (Hadrup et al., 2009; Stronen et al., 2016), or engineered chimeric antigen receptor (CAR) modified T cells (Benmebarek et al., 2019).

The calculation of percent cytotoxicity is based on the absolute counting beads method, which requires no-wash steps during sample preparation. It is necessary to label the target cells in advance. Therefore, it is inapplicable to perform the flow staining which requires a wash step.

TROUBLESHOOTING

Problem 1

Low yield, viability, or purity of PBMCs.

Potential Solution

Appropriate temperature is essential for the isolation of PBMCs. Higher temperature will decrease the density of Ficoll-Paque PLUS and cell viability, and increase the sedimentation rate of PBMCs, while lower temperature has the opposite effects. Therefore, it is pivotal to keep the temperature of all of reagents and centrifuge at 18°C–20°C. Besides, the vibration of the centrifuge rotor will cause perturbation of the gradient, thus it is necessary to use the brake-off mode for the deceleration. Moreover, begin the isolation of PBMCs as soon as possible when accessing the blood sample.

Problem 2

Low yield or viability of CD3⁺ T cells after separation.

Potential Solution

Inappropriate isolation temperature, low viability of PBMCs, inappropriate magnetic column, and formation of air bubbles or cell clumps will decrease the yield and viability of the CD3⁺ T cells. Always keep the temperature of all reagents and centrifuge at 4°C. Choose a suitable magnetic column for isolation, for example, for the total cells below 2×10^8 , use the MS column, and use LS column for the maximal total cell number up to the 2×10^9 . Work gently to avoid air bubbles. When working with cryopreserved PBMCs, treat the thawed cells with DNase I and passage it through a cell strainer to remove any cell clumps.

Problem 3

Low cytotoxicity of T cells toward tumor cells.

Potential Solution

The low tumoricidal effects mainly may be caused by the insufficient activation cells or exhaustion of T cells, scant number of effector cells, or inadequate incubation time of co-culture. To circumvent these factors, use the exact ratio of anti-CD3/CD28 Dynabeads. Restimulation of T cells when observed exhausted phenotype. Scale up the effector-tumor-ratio and elongate incubation time could augment cytotoxicity effects.

Problem 4

Large variation of the killing percentage among the triplicates of co-culture.

Potential Solution

The big error may arise due to, but not limited to, the following reasons, (i) inaccurate amount of absolute counting beads, (ii) washing the sample accidentally, (iii) the leftover of fluid inside the lateral wall of tubes, or (iv) an uneven mixture of cells and beads. Therefore, it is important to transfer all the cells from the culture plate to the flow tubes. Wash the wells twice rightly after harvest the cells and

collect all the fluid to the corresponding tubes. Vortex thoroughly of the counting beads before apply to the flow tube. If apply a small volume of counting beads, it is necessary to calculate the total amount of counting beads of all the samples, and then dilute it to a final volume of 100 μ L for each sample. Never wash the cells. Briefly centrifuge the flow tubes to take all the fluid to the bottom. Vortex the cells and beads mixture for at least 30 s, and then run the flow right away.

Problem 5

Low signal of bioluminescence.

Potential Solution

The reasons for low bioluminescence signal include: (i) low expression level of Luciferase in the tumor cells; (ii) low tumor burden in the mice; (iii) inappropriate exposure time; (iv) sudden death of the mice. To overcome these problems, make sure the Luciferase expression in the tumor cells. Follow up for a longer time for the tumor progression or scale up the initial dose of injected tumor cells. Set up two or three exposure time for capturing the images. Also, it is important to recognize the appropriate injection site for peritoneal injection and monitor the breath of mice in case of the hyperanesthesia of the mice, to avoid the unexpected death of the mice.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jianjun Chen, jjanchen@coh.org.

Materials Availability

The constructs and the stable cells are available upon request by contacting the Lead Contact.

Data and Code Availability

Not applicable.

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AUTHOR CONTRIBUTIONS

Y.L. and R.S. developed and optimized the protocol and wrote the manuscript. J.C. supervised the project and wrote the manuscript.

DECLARATION OF INTERESTS

A provisional patent was filed with J.C. and R.S. as inventors. J.C. is the scientific founder of Genovel Biotech Corp. and holds equities with the company.

REFERENCES

- Andersch, L., Radke, J., Klaus, A., Schwiebert, S., Winkler, A., Schumann, E., Grunewald, L., Zirngibl, F., Flemmig, C., Jensen, M.C., et al. (2019). CD171- and GD2-specific CAR-T cells potently target retinoblastoma cells in preclinical in vitro testing. *BMC Cancer* 19, 895.
- Baboo, J., Kilbride, P., Delahaye, M., Milne, S., Fonseca, F., Blanco, M., Meneghel, J., Nancekivill, A., Gaddum, N., and Morris, G.J. (2019). The impact of varying cooling and thawing rates on the quality of cryopreserved human peripheral blood T cells. *Sci. Rep.* 9, 3417.
- Bennebarek, M.R., Karches, C.H., Cadilha, B.L., Lesch, S., Endres, S., and Kobold, S. (2019). Killing mechanisms of chimeric antigen receptor (CAR) T cells. *Int. J. Mol. Sci.* 20.
- Bernin, H., Fehling, H., Marggraff, C., Tannich, E., and Lotter, H. (2016). The cytokine profile of human NKT cells and PBMCs is dependent on donor sex and stimulus. *Med Microbiol. Immunol.* 205, 321–332.
- Boudreau, G., Carli, C., Lamarche, C., Rulleau, C., Bonnaure, G., Neron, S., and Delisle, J.S. (2019). Leukoreduction system chambers are a reliable cellular source for the manufacturing of T-cell therapeutics. *Transfusion* 59, 1300–1311.
- Campeau, E., Ruhl, V.E., Rodier, F., Smith, C.L., Rahmberg, B.L., Fuss, J.O., Campisi, J., Yaswen, P., Cooper, P.K., and Kaufman, P.D. (2009). A versatile viral system for expression and depletion of proteins in mammalian cells. *PLOS ONE* 4 (8), e6529, <https://doi.org/10.1371/journal.pone.0006529>.
- Deng, M., Gui, X., Kim, J., Xie, L., Chen, W., Li, Z., He, L., Chen, Y., Chen, H., Luo, W., et al. (2018).

- LILRB4 signalling in leukaemia cells mediates T cell suppression and tumour infiltration. *Nature* 562, 605–609.
- Dull, T., Zufferey, R., Kelly, M., Mandel, R.J., Nguyen, M., Trono, D., and Naldini, L. (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* 72 (11), 8463–8471, <https://doi.org/10.1128/JVI.72.11.8463-8471.1998>.
- Dunsford, L.S., Thoires, R.H., Rathbone, E., and Patakas, A. (2020). A human in vitro T cell exhaustion model for assessing immuno-oncology therapies. In *Immuno-Oncology: Cellular and Translational Approaches*, S. Tan, ed. (Springer US), pp. 89–101.
- Gomes, A.Q., Correia, D.V., Grosso, A.R., Lanca, T., Ferreira, C., Lacerda, J.F., Barata, J.T., Silva, M.G., and Silva-Santos, B. (2010). Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gammadelta T cells. *Haematologica* 95, 1397–1404.
- Hadrup, S.R., Bakker, A.H., Shu, C.J., Andersen, R.S., van Veluw, J., Hombrink, P., Castermans, E., Thor, S.P., Blank, C., Haanen, J.B., et al. (2009). Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nat. Methods* 6, 520–526.
- Hakim, F.T., Memon, S.A., Cepeda, R., Jones, E.C., Chow, C.K., Kasten-Sportes, C., Odom, J., Vance, B.A., Christensen, B.L., Mackall, C.L., et al. (2005). Age-dependent incidence, time course, and consequences of thymic renewal in adults. *J. Clin. Invest.* 115, 930–939.
- Iyoda, T., Ushida, M., Kimura, Y., Minamino, K., Hayuka, A., Yokohata, S., Ehara, H., and Inaba, K. (2010). Invariant NKT cell anergy is induced by a strong TCR-mediated signal plus co-stimulation. *Int. Immunol.* 22, 905–913.
- Jahnke, S., Schmid, H., Secker, K.A., Einhaus, J., Duerr-Stoerzer, S., Keppeler, H., Schober-Melms, I., Baur, R., Schumm, M., Handgretinger, R., et al. (2019). Invariant NKT cells from donor lymphocyte infusions (DLI-INKTs) promote ex vivo lysis of leukemic blasts in a CD1d-dependent manner. *Front. Immunol.* 10, 1542.
- Kamala, T. (2008). An optimized immunomagnetic bead-based negative selection protocol for CD4 T-cell isolation from mouse lymph nodes and spleen. *Scand. J. Immunol.* 67, 285–294.
- Naylor, K., Li, G., Vallejo, A.N., Lee, W.W., Koetz, K., Bryl, E., Witkowski, J., Fulbright, J., Weyand, C.M., and Goronzy, J.J. (2005). The influence of age on T cell generation and TCR diversity. *J. Immunol.* 174, 7446–7452.
- Nelson, N., Lopez-Pelaez, M., Palazon, A., Poon, E., De La Roche, M., Barry, S., Valge-Archer, V., Wilkinson, R.W., Dovedi, S.J., and Smith, P.D. (2019). A cell-engineered system to assess tumor cell sensitivity to CD8(+) T cell-mediated cytotoxicity. *Oncoimmunology* 8, 1599635.
- Neron, S., Thibault, L., Dussault, N., Cote, G., Ducas, E., Pineault, N., and Roy, A. (2007). Characterization of mononuclear cells remaining in the leukoreduction system chambers of apheresis instruments after routine platelet collection: a new source of viable human blood cells. *Transfusion* 47, 1042–1049.
- Neubert, N.J., Sonesson, C., Barras, D., Baumgaertner, P., Rimoldi, D., Delorenzi, M., Fuertes, M.S., and Speiser, D.E. (2016). A well-controlled experimental system to study interactions of cytotoxic T lymphocytes with tumor cells. *Front. Immunol.* 7, 326.
- Pluhar, G.E., Pennell, C.A., and Olin, M.R. (2015). CD8(+) T cell-independent immune-mediated mechanisms of anti-tumor activity. *Crit. Rev. Immunol.* 35, 153–172.
- Salam, N., Rane, S., Das, R., Faulkner, M., Gund, R., Kandpal, U., Lewis, V., Mattoo, H., Prabhu, S., Ranganathan, V., et al. (2013). T cell ageing: effects of age on development, survival & function. *Indian J. Med. Res.* 138, 595–608.
- Stronen, E., Toebe, M., Kelderman, S., van Buuren, M.M., Yang, W., van Rooij, N., Donia, M., Boschen, M.L., Lund-Johansen, F., Olweus, J., et al. (2016). Targeting of cancer neoantigens with donor-derived T cell receptor repertoires. *Science* 352, 1337–1341.
- Su, R., Dong, L., Li, Y., Gao, M., Han, L., Wunderlich, M., Deng, X., Li, H., Huang, Y., Gao, L., et al. (2020). Targeting FTO suppresses cancer stem cell maintenance and immune evasion. *Cancer Cell* 38, 79–96.
- Vilalta, M., Jorgensen, C., Degano, I.R., Chernajovsky, Y., Gould, D., Noel, D., Andrades, J.A., Becerra, J., Rubio, N., and Blanco, J. (2009). Dual luciferase labelling for non-invasive bioluminescence imaging of mesenchymal stromal cell chondrogenic differentiation in demineralized bone matrix scaffolds. *Biomaterials* 30, 4986–4995.
- Wallace, D.L., Berard, M., Soares, M.V., Oldham, J., Cook, J.E., Akbar, A.N., Tough, D.F., and Beverley, P.C. (2006). Prolonged exposure of naive CD8+ T cells to interleukin-7 or interleukin-15 stimulates proliferation without differentiation or loss of telomere length. *Immunology* 119, 243–253.
- Weinberg, A., Song, L.Y., Wilkening, C., Sevin, A., Blais, B., Louzao, R., Stein, D., Defechereux, P., Durand, D., Riedel, E., et al. (2009). Optimization and limitations of use of cryopreserved peripheral blood mononuclear cells for functional and phenotypic T-cell characterization. *Clin. Vaccine Immunol.* 16, 1176–1186.
- Wunderlich, M., Mizukawa, B., Chou, F.S., Sexton, C., Shrestha, M., Sauntharajah, Y., and Mulloy, J.C. (2013). AML cells are differentially sensitive to chemotherapy treatment in a human xenograft model. *Blood* 121, e90–e97.