Chapter 4

Methods for Studying Mouse and Human Invariant Natural Killer T Cells

Yang Zhou, Yan-Ruide Li, Samuel Zeng, and Lili Yang

Abstract

Invariant natural killer T (iNKT) cells are a unique subset of T lymphocytes that recognize lipid antigens presented by nonpolymorphic major histocompatibility complex (MHC) I-like molecule CD1d. iNKT cells play essential roles in regulating immune responses against cancer, viral infection, autoimmune disease, and allergy. However, the study and application of iNKT cells have been hampered by their very small numbers (0.01–1% in mouse and human blood). Here, we describe protocols to (1) generate mouse iNKT cells from mouse mononuclear cells or from mouse hematopoietic stem cells engineered with iNKT T cell receptor (TCR) gene (denoted as mMNC-iNKT cells or mHSC-iNKT cells, respectively), (2) generate human iNKT cells from human peripheral blood mononuclear cells or from human HSC cells engineered with iNKT TCR gene (denoted as hPBMC-iNKT cells or hHSC-iNKT cells, respectively), and (3) characterize mouse and human iNKT cells in vitro and in vivo.

Key words Invariant natural killer T (iNKT) cell, CD1d, T cell receptor (TCR), Alpha-galactosylceramide (α-GalCer), Glycolipid, Gene engineering, Hematopoietic stem cell (HSC), Cancer immunotherapy

1 Introduction

Invariant natural killer T (iNKT) cells are a unique subpopulation of innate T lymphocytes that express both natural killer (NK) cell markers and a restricted αβ T cell receptor (TCR). The restricted TCR is comprised of a canonical invariant TCRα chain (Vα14-Jα18 in mice; Vα24-Jα18 in human) paired with a semi-variant TCRβ chain (mostly Vβ8.2 in mice; mostly Vβ11 in human) [1, 2]. The early developmental stages of iNKT cells are similar to classical MHC-restricted $CD4^+$ and $CD8^+$ conventional T (Tc) cells [2, 3]. Lymphoid precursor cells arising from hematopoietic stem

Yang Zhou and Yan-Ruide Li contributed equally to this work.

Chaohong Liu (ed.), *Invariant Natural Killer T-Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 2388,
https://doi.org/10.1007/978-1-0716-1775-5_4,

cells (HSCs) migrate to the thymus, undergo rearrangement of the TCRβ chain, and develop into CD4 and CD8 double-positive (DP) thymocytes. DP thymocytes then randomly rearrange their TCRα loci to generate intact TCR complexes expressed on the cell surface. However, unlike Tc cells that are selected by peptides presented on MHC-I or MHC-II of thymic epithelial cells, iNKT DP precursors are positively selected by glycolipids presented on CD1d expressed by DP thymocytes themselves [4]. The iNKT TCR-glycolipid-CD1d interaction, along with signals through the signaling lymphocytic activated molecules (SLAM) receptor family, provides co-stimulation for the further development of iNKT cells [3]. Owing to their unique developmental path, iNKT cells exit the thymus expressing a memory T cell phenotype. They further mature in the periphery through upregulating their expression of NK cell markers [5].

Functionally mature iNKT cells are powerful modulators of the immune response [4, 6–8]. Their most notable function is secreting copious amounts of cytokines upon stimulation, including T helper (Th)1-like (IFN-γ), Th2-like (IL-4, IL-13), Th17-like (IL-17, IL-22), and regulatory (IL-10) cytokines [4]. What cytokines are produced depends on the mechanism of cell activation, the location, and the iNKT cell subsets. iNKT cells also produce cytolytic proteins such as perforin and granzyme B and surface molecules involved in cytotoxicity such as Fas Ligand (FasL) and tumor necrosis factor α (TNF-α)-related apoptosis-inducing ligand (TRAIL) [9, 10]. Collectively, iNKT cells can profoundly influence many other immune cells, including dendritic cells, macrophages, neutrophils, NK cells, T cells, and B cells, thereby orchestrating the immune responses during infection, autoimmune disease [11], allergy [12], and cancer [9, 13, 14].

However, the extremely low number of iNKT cells, particularly in human peripheral blood (0.01–1% in healthy humans; 0.001–0.1% in cancer patients), is a significant obstacle for studying iNKT cell biology and developing iNKT cell-based therapies [5]. In our lab, we have developed methods to effectively generate large numbers of mouse and human iNKT cells through genetic engineering of HSCs (denoted as mHSC-iNKT cells and hHSC-iNKT cells, respectively [15–17]); we also routinely expand mouse iNKT cells from mouse mononuclear cells (denoted as mMNC-iNKT cells) and expand human iNKT cells from human peripheral blood mononuclear cells (denoted as hPBMC-iNKT cells) following established protocols with certain modifications [15, 17]. Here, we share our lab protocols on (1) generating mMNC-iNKT and mHSC-iNKT cells; (2) generating hPBMC-iNKT and hHSC-iNKT cells; (3) characterizing mouse and human iNKT cells with in vitro and in vivo assays.

2 Materials

Prepare all media in sterile hood (unless otherwise indicated).

2.1 Generation of Mouse iNKT Cells

2.1.1 Isolate and Expand mMNC-iNKT Cells

1. Mice: C57BL/6J (B6-WT) mouse.
2. Fetal bovine serum (FBS).
3. Phosphate-buffered saline (PBS).
4. 33% Percoll: 33% (vol/vol) of 100% Percoll, 67% (vol/vol) of 1× PBS solution.
5. 40% Percoll: 40% (vol/vol) of 100% Percoll, 60% (vol/vol) of 1× PBS solution.
6. 60% Percoll: 60% (vol/vol) of 100% Percoll, 40% (vol/vol) of 1× PBS solution.
7. Anti-mouse CD16/32 Fc block.
8. Anti-mouse CD19 microbeads.
9. Anti-mouse CD1d-Tetramer-PE.
10. Anti-PE microbeads.
11. 70 μm cell strainer.
12. Mouse iNKT culture medium: C10 medium supplemented with recombinant mouse IL-2 (final concentration 10 ng/ml) and IL-12 (final concentration 1 ng/ml).
13. Equipment: Magnetic beads separator (MACS), MACS Columns (LS column, LD column), water bath, sonicator, irradiator, pH meter.

2.1.2 Generate mHSC-iNKT Cells

1. Mice: C57BL/6J (B6-WT) mice.
2. Mouse iNKT TCR sequence [15].
3. 5-Fluorouracil (*see* **Note 1**).
4. Mouse iNKT retrovirus (*see* **Note 2**).
5. Human embryonic kidney cell line HEK293.T.
6. Polybrene.
7. Recombinant mouse IL-3.
8. Recombinant mouse IL-6.
9. Murine stem cell factor (SCF).
10. Antibiotics: sulfamethoxazole and trimethoprim.
11. Other reagents, material, and equipment were described in Subheading 2.1.

2.2 Generation of Human iNKT Cells

2.2.1 Isolate and Expand hPBMC-iNKT Cells

1. Human blood from healthy donors.
2. Ficoll-Paque Plus.
3. Anti-human iNKT MicroBeads.
4. Recombinant human IL-7.
5. Recombinant human IL-15.
6. Tris-buffered ammonium chloride buffer (TAC buffer or red blood cell lysis buffer): 0.16 M NH_4CL, 0.17 M Tris, ddH_2O.
7. MACS sorting buffer: phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2 mM EDTA.
8. C10 medium: RPMI1640 supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin/streptomycin/glutamine, 1% (vol/vol) MEM NEAA, 10 mM HEPES, 1 mM sodium pyruvate, and 50 μM β-ME.
9. Human iNKT culture medium: C10 medium supplemented with recombinant human IL-7 (final 10 ng/ml) and human IL-15 (final concentration 10 ng/ml).
10. α-GalCer medium: C10 medium supplemented with α-galactosylceramide (final concentration 5 μg/ml).

2.2.2 Generate hHSC-iNKT Cells

1. Mice: NOD.Cg-Prkdc^{SCID}Il2rg^{tm1Wjl}/SzJ (NOD/SCID/IL-2Rγ$^{-/-}$, NSG) mice.
2. Human CD34$^+$ hematopoietic stem cells (CD34$^+$ HSCs) (*see* **Note 3**).
3. Human fetal thymus tissues.
4. Human iNKT TCR sequences [17].
5. RetroNectin.
6. 2% BSA.
7. X-VIVO-15 serum-free medium.
8. Carprofen.
9. Recombinant human IL-3.
10. Human Flt3-Ligand (Flt3-L).
11. Human stem cell factor (hSCF).
12. Human thrombopoietin (TPO).
13. 6-well non-tissue culture treated plates.
14. Other reagents, materials, and equipment were described in Subheading 2.1.

2.3 Characterization of Mouse or Human iNKT Cells

2.3.1 Phenotype Analysis of Mouse or Human iNKT Cells

1. Antibodies (*see* Table 1).
2. Phorbol-12-myristate-13-acetate (PMA): 1 mg PMA dissolved in 400 μl DMSO.
3. Ionomycin: 1 mg ionomycin dissolved in 400 μl DMSO.
4. GolgiStop.
5. BD fixation/permeabilization solution kit.
6. Recombinant mouse and human IFN-γ (ELISA, standard).
7. Recombinant mouse and human IL-4 (ELISA, standard).
8. Anti-mouse and human IFN-γ (ELISA, capture).
9. Anti-mouse and human IFN-γ (ELISA, detection).
10. Anti-mouse and human IL-4 (ELISA, capture).
11. Anti-mouse and human IL-4 (ELISA, capture).
12. Nunc-Immuno ELISA plate.
13. ELISA coating buffer: 325 ml of 0.1 M $NaHCO_3$, 50 ml of 0.1 M Na_2CO_3, PH = 9.4, store at room temperature (RT).
14. ELISA borate buffered saline (BBS) dilution buffer: 6.07 g H_3BO_3 (0.1 M), 7.32 g NaCl (0.012 M), 20 g 2% BSA, 1 L ddH_2O, PH = 8, store at 4 °C.
15. ELISA wash buffer (20×): 1 M Tris (PH = 8.0), 163.5 g NaCl, 10 ml Tween-20.
16. Tetramethylbenzidine (TMB).
17. Streptavidin-HRP conjugate.
18. TMB reaction stop solution (1 M H_3PO_4): 68.2 ml 85% phosphoric acid, 1 L ddH_2O, store at RT.

2.3.2 Function Analysis of Mouse or Human iNKT Cells

1. Human CD14 microbeads.
2. NK isolation kit.
3. Mouse melanoma cell line B16.F10.
4. Human multiple myeloma cell line MM.1S.
5. Human multiple myeloma cell line MM.1S-FG and MM.1S-hCD1d-FG (*see* **Note 4**).
6. Human chronic myelogenous leukemia cancer cell line K562.
7. Human chronic myelogenous leukemia cancer cell line K562-FG (*see* **Note 5**).
8. Human melanoma cell line A375.
9. Human melanoma cell line A375-A2-Eso-FG (*see* **Note 6**).
10. D-luciferin.
11. Isoflurane.
12. Zeiss Stemi 2000-CS microscope (Carl Zeiss AG).
13. IVIS 100 imaging system (Xenogen/PerkinElmer).

Table 1
List of antibodies

Antibodies		
Anti-human CD45 (Clone H130)	BioLegend	CAT#304026, RFID: AB_893337
Anti-human TCRαβ (Clone I26)	BioLegend	CAT#306716, RRID: AB_1953257
Anti-human CD4 (Clone OKT4)	BioLegend	CAT#317414, RRID: AB_571959
Anti-human CD8 (Clone SK1)	BioLegend	CAT#344714, RRID: AB_2044006
Anti-human CD45RO (Clone UCHL1)	BioLegend	CAT#304216, RRID: AB_493659
Anti-human CD161 (Clone HP-3G10)	BioLegend	CAT#339928, RRID: AB_2563967
Anti-human CD69 (Clone FN50)	BioLegend	CAT#310914, RRID: AB_314849
Anti-human CD56 (Clone HCD56)	BioLegend	CAT#318304, RRID: AB_604100
Anti-human CD62L (Clone DREG-56)	BioLegend	CAT#304822, RRID: AB_830801
Anti-human CD14 (Clone HCD14)	BioLegend	CAT#325608, RRID: AB_830681
Anti-human CD1d (Clone 51.1)	BioLegend	CAT#350308, RRID: AB_10642829
Anti-mouse TCRβ (Clone 1B3.3)	BioLegend	CAT#156305, RRID: AB_2800701
Anti-mouse NK1.1 (Clone PK136)	BioLegend	CAT#108710, RRID: AB_313397
Anti-mouse CD62L (Clone MEL-14)	BioLegend	CAT#104411, RRID: AB_30566881
Anti-mouse CD44 (Clone IM7)	BioLegend	CAT#103012, RRID: AB_312963
Anti-mouse CD4 (Clone GK1.5)	BioLegend	CAT#100412, RRID: AB_312697
Anti-mouse CD8 (Clone 53-6.7)	BioLegend	CAT#100712, RRID: AB_312751
Anti-mouse CD3 (Clone 17A2)	BioLegend	CAT#100236, RRID: AB_2561456
Anti-mouse IFN-γ (Clone XMG1.2)	BioLegend	CAT#505809, RRID: AB_315403
Anti-mouse IL-4 (Clone 11B11)	BioLegend	CAT#504103, RRID: AB_315317
Anti-mouse CD1d (Clone K253)	BioLegend	CAT#140805, RRID: AB_10643277
Anti-human CD34 (Clone 581)	BD Biosciences	CAT#555822, RRID: AB_396151
Anti-human TCR Vα24-Jβ18 (Clone 6B11)	BD Biosciences	CAT#552825, RRID: AB_394478
Anti-human Vβ11	Beckman-Coulter	CAT#A66905
Human Fc Receptor Blocking Solution (TrueStain FcX)	BioLegend	CAT#422302
Mouse Fc Block (anti-mouse CD16/32)	BD Biosciences	CAT#553142, RRID: AB_394657
LEAF purified anti-human CD1d antibody (Clone 51.1)	BioLegend	CAT#350304
LEAF purified Mouse IgG2b, k isotype ctrl (Clone MG2b-57)	BioLegend	CAT#401212
Mouse Fluorochrome-conjugated mCD1d/PSC-57 tetramer	NIH Tetramer Core Facility	

3 Methods

3.1 Generation of mMNC-iNKT Cells

3.1.1 Prepare MNCs from Mouse Spleen and Liver (Fig. 1a)

1. Euthanize mice by CO_2.
2. Clean the skin by spraying with 70% ethanol. Dissect the mouse and collect the spleen, located on the left flank. The liver is preferably flushed of circulating blood prior to collection. To do so, shift the intestines away from the body to uncover the inferior vena cava, and use a 5 ml syringe to push approximately 5 ml of PBS through it. The liver should turn yellow/white as a result.
3. Harvested spleen and liver should be collected in separate tubes containing 3–5 ml sterile C10 medium (*see* **Note 7**).
4. In a sterile hood, disperse the liver and spleen into single cell suspensions by placing each tissue in a 70 μm cell strainer and mashing with a plunger from a 3 ml syringe. Rinse the plunger and cell strainer with C10 medium and transfer the cell suspension into a new 15 ml conical tube.
5. Liver and spleen samples are processed differently. **Steps 6–11** refer to processing the liver, while **steps 12** and **13** refer to processing the spleen.
6. Add 3 ml of 60% Percoll into a new 15 ml tube.
7. Spin down (600 × *g*) the liver cells and resuspend the cell pellet in 3 ml of 40% Percoll in PBS (*see* **Note 8**).
8. Gently layer the 40% Percoll cell suspension on top of the 60% Percoll. To do this, tilt the conical tube until it is almost horizontal and add the 40% suspension drop by drop. If performed correctly, one should see a sharp demarcation between the 40% and 60%.
9. Spin at 800 × *g* for 30 min with no brakes at RT (*see* **Note 9**).
10. Aspirate the floating debris. There should be a thin layer of cells around the 3 ml line. Collect those cells while avoiding the red blood cells found at the bottom of the tube.
11. Add 5 ml C10 medium to the collection and mix well. Spin down for 5 min at 4 °C. Aspirate the supernatant and resuspend in 5 ml C10 medium.
12. Spin down (600 × *g*) the spleen cells and resuspend in 5 ml TAC buffer at room temperature for 10–20 min to lyse the red blood cells.
13. Add an additional 5 ml C10 medium to neutralize the buffer, spin down (600 × *g*). Aspirate the supernatant and resuspend the pellet with 5 ml C10 medium. Cells clumps may be observed. Filter through a 70 μm cell strainer to remove dead cell clumps.

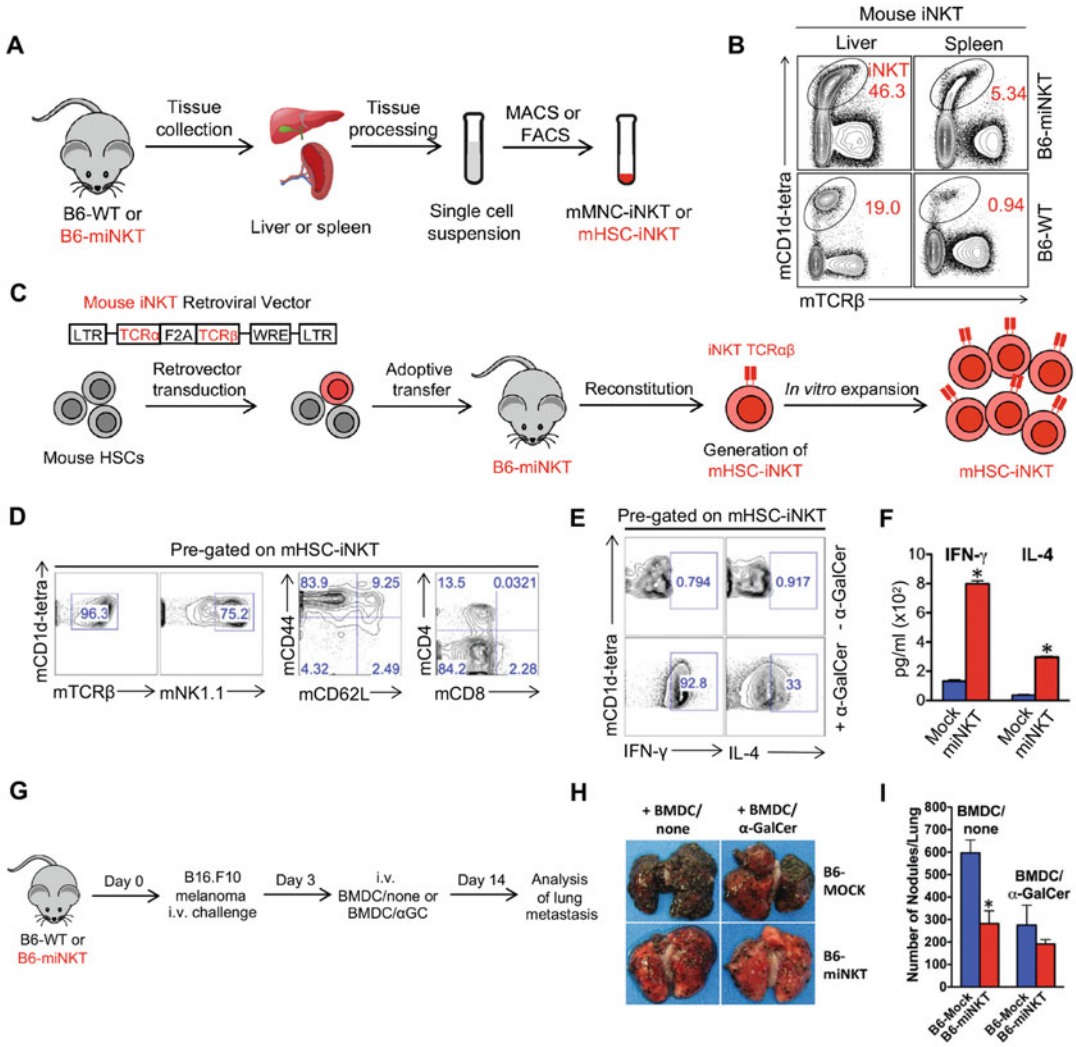

Fig. 1 Generation and characterization of mouse iNKT cells. (**a**) Diagram depicting the isolation of mMNC-iNKT cells and mHSC-iNKT cells. (**b**) FACS detection of mouse iNKT cells in liver and spleen of B6-miNKT mice or B6-WT mice. (**c**) Experimental design for generating mHSC-iNKT cells in a B6-miNKT mouse model. (**d**) FACS detection of the surface markers of mHSC-iNKT cells. These iNKT cells were detected in the liver of B6-miNKT mice for up to 6 months after HSCs adoptive transfer. (**e**, **f**) Functionality of mHSC-iNKT cells tested in vitro. Spleen cells collected from B6-miNKT mice were cultured in vitro in the presence of α-GalCer (100 ng/ml) (**e**) FACS detection of intracellular cytokine production in mHSC-iNKT cells 3 days post α-GalCer stimulation. (**f**) ELISA analysis of cytokine production of mHSC-iNKT cells in the cell culture medium 3 days post α-GalCer stimulation. (**g**–**i**) Study in vivo antitumor efficacy of mHSC-iNKT cells using an B16.F10 melanoma lung metastasis mouse model. (**g**) Experimental design. (**h**) Photos of lung tumor nodules. Representative of two experiments. (**i**) Enumeration of lung tumor nodules. Data were presented as the mean ± SEM. $^*P < 0.01$, by Student's *t* test. (Note that **d**–**i** were reproduced from Ref. 15 with permission from NAS, copyright (2015) National Academy of Sciences)

14. Count live cell numbers. Cell resuspension can be kept at 4 °C, ready for sorting or FACS staining (*see* **Note 10**).

3.1.2 Magnetic Separation of mMNC-iNKT Cells

1. Resuspend MNCs from Subheading 3.1.1 in 2% FBS/PBS buffer.
2. Centrifuge the cell mixture, aspirate the supernatant, and resuspend in 100 μl of 2% FBS/PBS.
3. Add 10 μl of anti-mouse CD16/CD32 mAb to block nonspecific binding to Fcγ receptors and incubate for 5 min at 4 °C.
4. Add 20 μl of anti-mouse CD19 microbeads and incubate for 15 min at 4 °C.
5. Meanwhile, prepare a LD column and equilibrate with 2 ml of 2% FBS/PBS buffer.
6. Wash the cells with 2% FBS/PBS buffer and centrifugation at 600 × *g* for 5 min at 4 °C.
7. Resuspend cells in 500 μl of buffer and add to the LD column.
8. Collect the $CD19^-$ fraction into a clean 15 ml conical tube. Wash the column twice with 1 ml of 2% FBS/PBS each time and keep collecting the flow through.
9. Spin down the $CD19^-$ fraction and stain with 20 μl of anti-mouse CD1d-Tetramer-PE in 30 μl of 2% FBS/PBS for 20 min on ice.
10. Wash the cells with 2% FBS/PBS buffer and centrifugation at 600 × *g* for 5 min at 4 °C.
11. Stain with 20 μl of anti-PE microbeads in 100 μl of 2% FBS/PBS for 15 min on ice.
12. Meanwhile, prepare another LD column and equilibrate with 2 ml of 2% FBS/PBS.
13. Wash the cells with 2% FBS/PBS buffer and centrifuge at 600 × *g* for 5 min at 4 °C. Resuspend cells in 500 μl of buffer.
14. Load cell suspension into LD column. Mouse iNKT cells will bind to the column by positive selection. Wash the column twice with 1 ml of 2% FBS/PBS each time. The flow through can be discarded but can also be kept for troubleshooting.
15. Remove the LD column from the magnetic field and elute the cells from column.
16. The purity of the iNKT cells can be checked by FACS staining and can be further improved via FACS sorting (Fig. 1b).

3.1.3 In Vitro Expansion of mMNC-iNKT Cells

1. Count the number of mMNC-iNKT cells from Subheading 3.1.2.
2. Seed cell at 2×10^6 cells per well in the C10 medium, with or without the addition of α-GalCer (final concentration 100 ng/ml) for 5 days (*see* **Note 11**).
3. On day 3 and day 5, collect cells and run assays for mMNC-iNKT cell expansion using flow cytometry.

3.2 Generation of Mouse HSC-iNKT Cells (mHSC-iNKTs)

3.2.1 Generate HSC-iNKT Mouse (See **Note 12**) (Fig. 1c)

1. Day 0, treat B6 mice with 5-fluorouracial (250 μg per gram body weight).
2. Day 5, harvest bone marrow (BM) cells from mouse and culture the cells for 4 days in BM cell culture medium containing recombinant mouse IL-3 (20 ng/ml), IL-6 (50 ng/ml), and SCF (50 ng/ml).
3. Day 7 and 8, BM cells were spin-infected with retroviruses (*see* **Note 13**) supplemented with 8 μg/ml of polybrene, at 770 × *g*, 30 °C for 90 min.
4. Day 9, BM cells were collected and intravenously injected into B6 recipients that had received 1200 rads of total body irradiation (~1–2 × 10^6 transduced BM cells per recipient) (*see* **Note 14**).
5. The BM recipient mice were maintained on the combined antibiotics sulfamethoxazole and trimethoprim oral suspension in a sterile environment for 6–8 weeks until analysis or use for subsequent experiments.

3.2.2 Isolate and Expand mHSC-iNKT Cells (Refer Subheading 3.1)

1. Purify mHSC-iNKT cells following the steps of magnetic separation of mMNC-iNKT cells (refer Subheading 3.1.2).
2. Count the number of mHSC-iNKT cells, seed cells at 2 × 10^6 cells per well in the C10 medium, with or without the addition of α-GalCer (final concentration 100 ng/ml) for 5 days.
3. On day 3 and day 5, collect cells and run assays for mHSC-iNKT cell expansion using flow cytometry.

3.3 Generation of hPBMC-iNKT Cells

3.3.1 Isolate PBMCs from Human Peripheral Blood (Fig. 2a)

1. Obtain peripheral blood from healthy donors in blood collection tubes with heparin 1000 U/ml.
2. Centrifuge at 400 × *g* for 15 min with no brakes at RT.
3. Aspirate the supernatant and resuspend the cell pellet with PBS (10–12 ml/tube).
4. Transfer the mixture to a 50 ml conical tube. Wash once more using RT PBS (10–12 ml/tube).
5. Aspirate the supernatant and resuspend with 14 ml PBS.
6. Gently layer 14 ml of room temperature Ficoll on top of the mixture, using a 25 ml pipette (*see* **Note 15**).
7. Centrifuge at 970 × *g* for 20 min with no brakes at RT.
8. Aspirate the upper PBS layer.
9. Using a 5 ml pipette, carefully collect the PBMCs at the interface.
10. Wash with 10–20 ml PBS and centrifuge at 400 × *g* for 7 min at RT.

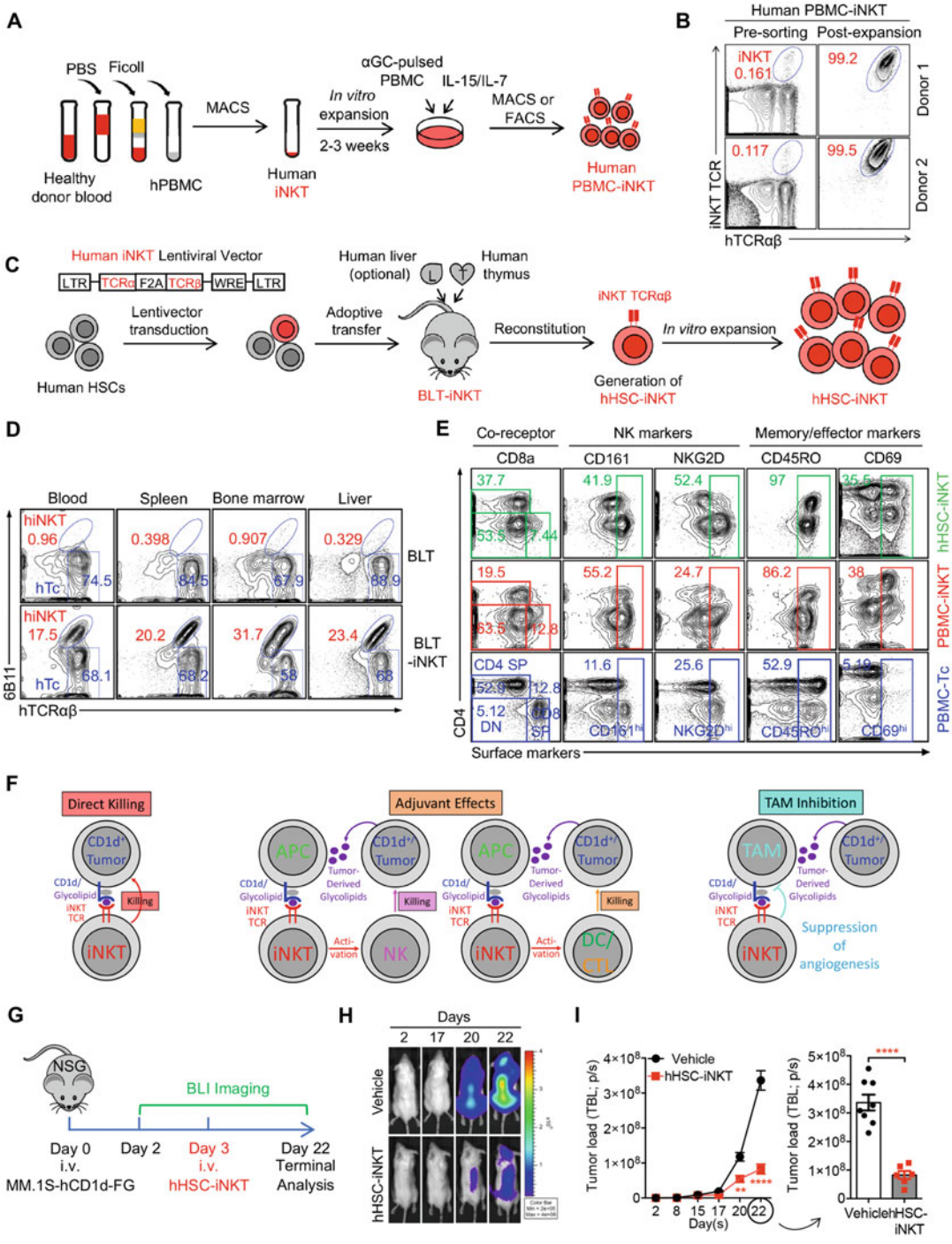

Fig. 2 Generation and characterization of human iNKT cells. (**a**) Diagram depicting the isolation and in vitro expansion of hPBMC-iNKT cells. (αGC: α-GalCer.). (**b**) FACS detection of hPBMC-iNKT cells before or after MACS sorting using anti-human iNKT microbeads. (**c**) Experimental design to generate hHSC-iNKT cells in a bone marrow-liver-thymus (BLT) humanized mouse model. (**d**) FACS detection of hHSC-iNKT cells in control BLT and BLT-iNKT mice tissues, at week 20 post-HSC transfer. Control BLT mice were generated by

11. Aspirate the supernatant and resuspend the PBMC pellet in 10 ml TAC buffer for 10–20 min at RT.
12. Centrifuge at 600 × *g* for 5 min and remove the supernatant.
13. Wash PBMCs with C10 medium once and resuspend cells in 10 ml of C10 medium.
14. Count live cell numbers.

3.3.2 Magnetic Separation of hPBMC-iNKT Cells

1. Count the number of cells in PBMC sample (*see* **Note 16**).
2. Centrifuge cell suspension at 300 × *g* for 10 min.
3. Aspirate supernatant completely and resuspend cell pellet in 400 μl of MACS sorting buffer per 1×10^8 total cells.
4. Add 100 μl of anti-human iNKT microbeads per 1×10^8 total cells. Mix well and incubate for 15 min in the refrigerator (2–8 °C).
5. Wash cells by adding 1–2 ml of buffer per 1×10^8 cells and centrifuge at 300 × *g* for 10 min. Aspirate supernatant completely and resuspend up to 1×10^8 cells in 500 μl of buffer.
6. Place column in the magnetic field of a suitable MACS separator.
7. Equilibrate column by rinsing with the appropriate amount of buffer (LS: 3 ml).
8. Apply cell suspension into the column. Collect flow-through containing unlabeled cells (*see* **Note 17**).
9. Wash column with the appropriate amount of buffer. Collect unlabeled cells that pass through and combine with the effluent (LS: 3 × 3 ml).
10. Remove column from the separator and place it on a clean 15 ml conical tube.
11. Pipette 3 ml of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column (*see* **Note 18**).
12. Check the purity of human iNKT cells with FACS staining.

Fig. 2 (continued) adoptively transferring mock-transduced human HSCs into NSG mice engrafted with human thymus. (**e**) FACS detection of the surface markers of hHSC-iNKT cells isolated from the spleen of BLT-iNKT mice. Human PBMC-iNKT cells and human PBMC-derived conventional αβ T (PBMC-Tc) cells were included as controls. (**f**) Diagram showing the possible mechanisms used by human iNKT cells to attack tumor cells. APC, antigen presenting cell; NK, natural killer cell; DC, dendritic cell; CTL, cytotoxic T lymphocyte; TAM, tumor-associated macrophage. (**g–i**) Study in vivo antitumor efficacy of hHSC-iNKT cells using an MM.1S-hCD1d-FG human MM xenograft NSG mouse model. (**g**) Experimental design. (**h**) BLI images showing tumor burden in experimental mice over time. Representative of three experiments. (**i**) Quantification of (**h**) ($n = 6$–8). Data were presented as the mean ± SEM. ****$P < 0.0001$, by Student's *t* test. (Note that **d–i** were reproduced from Ref. 17 with permission from Elsevier, copyright (2019) Elsevier)

3.3.3 In Vitro Expansion of hPBMC-iNKT Cells

1. Count the number of PBMC-iNKT cells from magnetic separation. (Usually around 5×10^8 PBMC will yield $0.5–2 \times 10^6$ iNKT cells.)
2. Load a portion of the negative fraction ($1–2 \times 10^8$ per 5 ml) with α-GalCer (5 μg/ml) (*see* **Note 19**) and irradiate (~70% yield, 6000 rads); freeze down the remaining negative portion.
3. Seed cells at 1×10^6 iNKT: 2×10^6 α-GalCer-pulsed PBMC per 3 ml C10 medium per well of a 6-well plate. Add IL-7 and IL-15 at 10 ng/ml to the culture.
4. Monitor cell growth daily. As cells reach saturation, add C10 medium containing human IL-7 and IL-15 at 10 ng/ml and split cultures.
5. Cells can expand five- to tenfold and reach ~80% iNKT cell confluency during the first week. At day 7, cells can be restimulated (repeat **steps 2** and **3**). Cells can expand around tenfold per stimulation.
6. Take a small aliquot of iNKT culture for FACS staining (Fig. 2b).
7. The culture reaches >95% iNKT cell during the second week. iNKT cells can be frozen down and kept in liquid nitrogen for long-term storage (*see* **Note 20**).

3.4 Generation of Human HSC-iNKT Cells

3.4.1 Generate HSC-iNKT Humanized Mouse (Fig. 2c) (See ***Note 21***)

1. Day 1, thaw and prestimulate $CD34^+$ PBSCs.
   (a) Coat 6-well non-tissue culture treated plates with RetroNectin (RN, 20 μg per vial in PBS) at RT for 2 h.
   (b) Aspirate and replace with 1 ml of 2% BSA for 30 min at RT.
   (c) Aspirate and replace with 2 ml PBS (*see* **Note 22**).
   (d) Thaw $CD34^+$ PBSC using X-VIVO-15 medium, spin at $300 \times g$ for 7 min.
   (e) Aspirate supernatant and resuspend in 5 ml of X-VIVO-15 medium and count cell number.
   (f) Spin down at $300 \times g$ for 7 min and aspirate supernatant.
   (g) Prepare 10 ml of X-VIVO-15 medium supplemented with hSCF (50 ng/ml), hFLT3L (50 ng/ml), hTPO (50 ng/ml), and hIL-3 (10 ng/ml).
   (h) Resuspend $CD34^+$ cells in X-VIVO-15/hSCF/hFLT3L/hTPO/hIL-3 medium (1×10^6 cells/ml).
   (i) Aspirate PBS in RN-coated well and seed the cells (1×10^6 per well).
   (j) Incubate at 37 °C, 5% CO_2.
2. Day 2, transduce $CD34^+$ PBSCs with lentivirus (*see* **Note 23**).

(a) Thaw concentrated virus supernatant on ice.

(b) Pipet thawed supernatant and add directly to well (*see* **Note 24**). Rock plate gently to mix.

(c) Incubate cells at 37 °C, 5% CO_2 for 24 h.

3. Day 3, prepare thymus pieces and intravenously inject transduced PBSCs to NSG mice.
   (a) Prepare fetal thymus fragments and irradiate with 500 rads (*see* **Note 25**).
   (b) Incubate irradiated thymus in C10 medium with antibiotics until surgery. Make sure to wash thymus thoroughly and keep on ice until surgery.
   (c) Irradiate NSG mice with 270 rads.
   (d) Harvest and count transduced human $CD34^+$ cells 24 h post transduction, and then resuspend in X-VIVO-15 medium (*see* **Note 26**).
   (e) Implant thymus pieces under the kidney capsule of pre-irradiated NSG mouse. Additionally, give retro-orbital injections of transduced PBSCs to each mouse.
   (f) Suture and staple the incision.
   (g) Subcutaneously inject 300 μl of 1:100 carprofen diluted in PBS (*see* **Note 27**).
4. Day 4, 5, daily injection with 300 μl of 1:100 carprofen diluted in PBS.
5. Day 7, remove the staples and monitor the conditions of BLT-iNKT mice.
6. Starting from week 6 post injection, bleed mice monthly and check human cell reconstitution by FACS staining (*see* **Note 28**).

3.4.2 hHSC-iNKT Cells from Humanized Mouse (Fig. 2d)

1. Euthanize mice by CO_2.
2. Place mouse in dorsal recumbency and clean the skin by spraying with 70% ethanol.
3. Cut the skin and expose both the abdomen and chest.
4. Puncture the heart with 26-gauge on 1 ml syringe to collect blood in a heparin-coated collection tube.
5. Collect the lung, liver, spleen, and bone marrow from humanized mouse and store the tissues on ice.
6. Disperse tissues into mononuclear cell suspension:
   (a) For blood, incubate in 5 ml TAC buffer for 20 min at RT; spin down to remove supernatant and resuspend in 1 ml C10 medium. Store at 4 °C. Sample is ready for staining (refer Subheading 3.5.1).

(b) For spleen, lung, and liver, mash tissues in C10 medium through a 70 μm cell strainer using plungers from 5 ml syringes. Collect the single cell suspension in a 15 ml conical tubes, spin down to remove supernatant, and resuspend in 14 ml 33% Percoll in PBS (spleen samples can skip Percoll separation and directly proceed to TAC lysis). Spin at 800 × *g* for 30 min with no brakes at RT. Aspirate the supernatant and resuspend the pellet in 5 ml TAC buffer. Incubate for 10 min at RT, and then add additional C10 medium and filter through cells strainer. Spin down and resuspend in fresh C10 medium. Store samples at 4 °C. Samples are ready for staining or cryopreservation (refer to Subheading 3.5.1).

(c) For bone marrow, use forceps to hold leg bones over a 15 ml conical tube and flush with C10 medium using 25-gauge needle fitted onto a 10 ml syringe. Spin down to remove the supernatant and resuspend in 10 ml TAC buffer. Incubate for 10 min at RT, and then filter through a 70 μm cell strainer. Spin down, aspirate the supernatant, and wash with 2 ml C10 medium. Resuspend the sample in C10 medium, and store at 4 °C. Sample is ready for staining or cryopreservation (refer Subheading 3.5.1).

3.4.3 In Vitro Expansion of hHSC-iNKT Cells

1. Healthy donor PBMCs were loaded with α-GalCer (by culturing 1×10^8 PBMCs in 5 ml C10 medium containing 5 μg/ml α-GalCer for 1 h in 6-well TC plate), irradiated at 6000 rads, and then used to stimulate iNKT cells.
2. To expand iNKT cells, pooled hHSC-iNKT humanized mouse tissue cells were mixed with α-GalCer-pulsed PBMCs (ratio 1:1 or 1:1.5; e.g. 1×10^6 iNKT tissue cells were mixed with 1.5×10^6 irradiated α-GalCer-pulsed PBMCs) and cultured in C10 medium for 7 days. Cells were plated in 6-well plate (2.5×10^6/ml, 3 ml/well). Recombinant human IL-7 (10 ng/ml) and IL-15 (10 ng/ml) were added to cell cultures starting from day 2. Cells were split 1:2 once confluent (about every 2–3 days).
3. On day 7, cell cultures were collected and iNKT cells were sorted out using flow cytometry (identified as $hCD45^+hTCR\alpha\beta^+6B11^+$ cells).
4. The sorted iNKT cells (>99% purify based on flow cytometry analysis) were expanded further with α-GalCer-pulsed PBMCs and human IL-7/IL-15 for another 7 to 14 days (*see* **Note 29**).
5. Expanded iNKT cells were aliquoted and frozen in LN storage tanks (e.g., 1×10^7 cells per vial for in vitro assay and 1×10^8 cells per vial for in vivo assay).

3.5 Characterization of Mouse or Human iNKT Cells

3.5.1 iNKT Cell Phenotype Analysis

1. Surface and intracellular marker staining (Figs. 1d, e, and 2e):
   (a) Aliquot cells into labeled FACS tubes.
   (b) Wash cells with 1 ml C10 media, spin down, and aspirate the supernatant.
   (c) Wash cells with 1 ml PBS, spin down, and aspirate the supernatant.
   (d) Resuspend cells in 50 μl PBS with human FcR Block and Fixable Viability Dye e506 (*see* **Note 30**).
   (e) Incubate cells at 4 °C for 15 min shielded from light.
   (f) Wash cells with 1 ml PBS, spin down, and aspirate the supernatant.
   (g) Resuspend cells in 50 μl PBS with antibody cocktails.
   (h) Incubate cells at 4 °C for 15 min shielded from light.
   (i) Wash cells with 1 ml PBS, spin down, and aspirate the supernatant.
   (j) For surface staining, resuspend the cell pellet in 100–200 μl PBS for flow cytometry.
   (k) For intracellular staining, add 250 μl of BD fixation/permeabilization buffer to the cell pellet.
   (l) Incubate cells at 4 °C for 20–30 min, shielded from light.
   (m) Spin at 600 *g* for 5 min, and aspirate the supernatant.
   (n) Wash twice with BD washing buffer.
   (o) Spin and resuspend the cell pellet in 50 μl intracellular antibodies cocktail.
   (p) Incubate cells at 4 °C for 30 min shielded from light.
   (q) Wash cells twice with 1 ml wash buffer.
   (r) Resuspend cells in 100–200 μl PBS for flow cytometry.
2. Stimulate cytokine production (PMA/Ionomycin stimulation):
   (a) Resuspend cells at 1×10^6/ml in C10 medium containing PMA (final concentration 50 ng/ml) and ionomycin (final concentration 500 ng/ml).
   (b) Transfer 1 ml of cells into capped FACS tubes.
   (c) Add GolgiStop to cells (4 μl GolgiStop per 6 ml of C10 medium) and tightly close caps on FACS tubes.
   (d) Incubate at 37 °C, 5% CO_2 for 4–6 h.
   (e) Samples are ready for FACS staining (refer Subheading 3.5.1, **step 1**).
3. ELISA (following standard protocol from BD bioscience) (Fig. 1f).

(a) Coat Nunc Immunoplates with purified capturing antibody diluted in ELISA coating buffer. Add 50 μl/well and incubate for 2 h at 37 °C or overnight at 4 °C.

(b) Wash plate four times with ELISA wash buffer.

(c) Block plate with 100 μl/ well of ELISA BBS buffer. Incubate for 30 min at 37 °C or overnight at 4 °C.

(d) Wash plate four times with ELISA wash buffer.

(e) Add samples at 25 μl or 50 μl per well. Incubate for 3 h at 37 °C or 4 °C overnight.

(f) Wash plate four times with ELISA wash buffer.

(g) Add 50 μl of the biotinylated detection antibody diluted in BBS solution buffer. Incubate for 45 min at RT.

(h) Wash plate four times with ELISA wash buffer.

(i) Add 50 μl/ well of the streptavidin-HRP, diluted 1:1000 in BBS dilution buffer. Incubate for 30 min at RT, shielded from light.

(j) Wash plate eight times with ELISA wash buffer.

(k) Mix the TMB developing solution and add 50 μl/ well. Incubate at RT.

(l) Monitor the blue color change and stop reaction by adding 50 μl/well of TMB reaction stop solution.

(m) Read absorbance at 450 nm within 30 min.

3.5.2 iNKT Cell Function Analysis

1. mHSC-iNKT cell in vivo antitumor efficacy study—mouse B16 melanoma lung metastasis mouse model [15] (Fig. 1g–i):
   (a) C57BL/6 J (B6) mice received intravenous (i.v.) injection of 0.5–1 × 10^6 B16.F10 melanoma cells to model lung metastasis over the course of 2 weeks.
   (b) On day 3 post tumor challenge, the experimental mice received i.v. injection of 1 × 10^6 bone marrow-derived dendritic cells (BMDCs) that were either unloaded or loaded with α-GalCer.
   (c) On day 14, mice were humanely euthanized, and their lungs were collected and analyzed for melanoma metastasis by counting tumor nodules.
2. hHSC-iNKT cell tumor-attacking mechanism study [17] (Fig. 2f):
   (a) In vitro direct tumor cell killing assay. Human multiple myeloma cell line MM.1S was used. MM.1S-FG or MM.1S-hCD1d-FG tumor cells (5–10 × 10^3 cells per well) were co-cultured with hHSC-iNKT cells (ratio 1:1, 1:2, 1:5, and 1:10) in Corning 96-well clear bottom black plates for 24–48 h, in X-VIVO™ 15 medium with or

without the addition of α-GalCer (100 ng/ml). At the end of culture, live tumor cells were quantified by adding 150 mg/ml of D-luciferin to cell cultures and reading out luciferase activities. In order to verify CD1d-dependent tumor killing mechanism, we blocked CD1d by adding 10 mg/ml LEAF™ purified anti-human CD1d antibody or LEAF™ purified mouse IgG2b κ isotype control antibody to tumor cell cultures at least 1 h prior to adding hHSC-iNKT cells. At the end of culture, live tumor cells were quantified by adding D-Luciferin to cell cultures and reading out luciferase activities.

(b) In vitro NK adjuvant effect assay. Primary human NK cells were isolated from healthy donor PBMCs through an NK Cell Isolation Kit according to the manufacturer's instructions. K562-FG cells (5×10^4 cells per well) were co-cultured with NK cells and hHSC-iNKT cells (at ratio of 1: 2: 2) in Corning 96-well clear bottom black plates for 24 h, in C10 medium with or without α-GalCer-pulsed irradiated PBMCs as antigen-presenting cells (APCs). Live tumor cells were quantified by adding D-luciferin (150 mg/ml) to the cell cultures and reading out luciferase activities.

(c) In vitro dendritic cells (DC)/cytotoxic T lymphocyte (CTL) adjuvant effect assay. $CD1d^+$/HLA-$A2^+$ human monocyte-derived dendritic cells (MoDCs) were generated by isolating $CD14^+$ monocytes from HLA-$A2^+$ healthy donor PBMCs using anti-human CD14 beads, followed by a 4-day culture in R10 medium supplemented with recombinant human GM-CSF (100 ng/ml) and IL-4 (20 ng/ml). The NY-ESO-1 specific $CD8^+$ human CTLs were co-cultured with $CD1d^+$/HLA-$A2^+$ MoDCs in C10 medium for 3 days, with or without hHSC-iNKT cells (cell ratio 1:1:1) and α-GalCer (100 ng/ml). Tumor-killing potential of ESO-T cells was measured by adding A375-A2-ESO-FG tumor cells (1:1 ratio to input ESO-T cells) to the ESO-T/MoDC co-culture 24 h post co-culture setup and quantifying live tumor cells by luciferase activity reading in another 24 h (*see* **Note 31**).

(d) In vitro macrophage inhibition assay. $CD14^+$ monocytes were isolated from healthy donor PBMCs, followed by co-culturing with hHSC-iNKT cells (ratio 1:1) for 24–48 h in C10 medium with or without the addition of α-GalCer (100 ng/ml). At the end of culture, cells were collected for flow cytometry analysis.

3. hHSC-iNKT cell in vivo antitumor efficacy study—MM.1S human multiple myeloma xenograft NSG mouse model [17] (Fig. 2g–i):
   (a) NSG mice were pre-conditioned with 175 rads of total body irradiation and inoculated with 0.5–1 $\times$ 10^6 MM.1S-hCD1d-FG or MM.1S-FG cells intravenously (day 0) to develop multiple myeloma over the course of about 3 weeks.
   (b) Three days post-tumor inoculation (day 3), mice received i.v. injection of vehicle (PBS) or 1 $\times$ 10^7 hHSC-iNKT cells. Recombinant human IL-15 was intraperitoneally injected to experimental animals to support the peripheral maintenance of hHSC-iNKT cells twice per week starting from day 3 (500 ng per animal per injection).
   (c) The tumor burden was monitored twice per week by bioluminescence (BLI) measurement.
   (d) At around week 3, mice were humanely euthanized (refer to Subheading 3.4.2), and tissues (peripheral blood, spleen, liver, and bone marrow) were collected for flow cytometry analysis.

4 Notes

1. 5-Fluorouracil (5-FU) is a chemotherapy drug used to treat cancer. 5-FU can inhibit thymidylate synthetase function during pyrimidine synthesis. The carcinogenicity and acute toxicity of 5-FU require proper handling from lab personnel.
2. The generation of mouse iNKT TCR gene delivery retroviral vector was described in our previous publication [15].
3. Human $CD34^+$ HSCs are commercially available from Hema-Care Corporation (Northridge, California, USA).
4. Human multiple myeloma (MM) cell line MM.1S, human chronic myelogenous leukemia cancer cell line K562, and human melanoma cell line A375 were all purchased from the American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and cultured in ATCC recommended media. The stable MM.1S-FG and MM.1S-hCD1d-FG tumor cell lines were engineered by transducing the parental MM.1S cell line with the lentiviral vectors encoding the intended gene(s) to overexpress human CD1d and/or firefly luciferase and enhanced green fluorescence protein (GFP) dual-reporters (FG). $CD1d^+$ and/or GFP^+ cells were sorted by flow cytometry 72 h post viral transduction to generate stable cell lines.
5. The stable K562-FG tumor cell line was engineered by transducing the parental K562 cell line with lentiviral vectors

encoding FG. GFP^+ cells were sorted by flow cytometry post 72 h of virus transduction to generate stable cell line.

6. The stable A375-A2-Eso-FG tumor cell line was engineered by transducing the parental A375 cell line with lentiviral vectors encoding human HLA-A2.1, human NY-ESO-1, and FG.
7. Keeping tissues on ice during processing improves the viability of cells.
8. 33%, 40%, and 60% Percoll can be prepared beforehand and stored long term at 4 °C. However, Percoll must be warmed back to RT before usage.
9. Centrifuge must be equilibrated to RT before starting and the centrifuge brakes must be turned off. Excessive deceleration can remix the separating layers.
10. Cells from spleen and liver can be combined for in vitro expansion if needed. Cells from multiple mice can be combined if needed.
11. mMNC-iNKT cells may be expanded and cultured in vitro for up to 3 weeks using repetitive stimulations with anti-CD3ε and anti-CD28 every 7–8 days [18]. α-GalCer stimulation leads to apoptosis and is not suitable for long-term expansion of mouse iNKT cells [18].
12. We have established a B6-miNKT mouse model through genetic engineering of hematopoietic stem cells [15] to produce large numbers of iNKT cells. Compared to B6-WT mice, B6-miNKT mice provide a significantly higher yield of iNKT cells.
13. Spin infections on two sequential days increase retrovirus transduction rate. Preferably, the second infection should be performed within 12–15 h after the first one to infect cells at different stages of the cell cycle.
14. For a secondary BM transfer, fresh whole BM cells harvested from the primary BM recipients are intravenously injected into secondary B6 recipient mice that had received 1200 rads of total body irradiation (~1×10^7 total BM cells per recipient). Details were described in previous publications [15, 16].
15. Tilt the tube and bring the pipette close to surface of the blood/PBS mixture. Slowly pipette out 1–2 ml. Then detach the pipette to allow gravity to dispense the remainder of the Ficoll. When the flow stops, reattach the pipette to push out any remaining Ficoll before removing the pipette from the conical tube. Be extremely careful at this step to make sure that the interface is not disturbed.
16. Choose the right column to use based on the sample cell number. For example, if there are ~2×10^8 total PBMCs, use one LS column.

17. Do not let the column dry out and avoid adding bubbles into the column. Bubbles inside the column can interfere with the sample and decrease selection efficacy.
18. To increase the purity, the eluted fraction can be enriched over a second column. Repeat the magnetic separation procedure as described if needed.
19. α-GalCer glycolipid and DMSO are immiscible at RT. To prepare the stock α-GalCer (1 μg/μl), add the proper volume of DMSO into the α-GalCer powder and heat it at 80 °C in a water bath for 10 min, followed by 10 min of sonication at 50 °C. Then, vortex the vial for full 2 min until the solution turns clear. Aliquot it into glass vials and store them in a −20 °C freezer. To prepare the α-GalCer working solution (5 μg/ml), heat the aliquot at 80 °C for 5 min followed by 5 min of sonicating at 50 °C. Then vortex the aliquot for a full 60 s and add 200 μl of pre-warmed C10 medium. Sonicate for another 5 min and vortex for 60 s. Add the rest of the pre-warmed C10 medium to make the final concentration 5 μg/ml. α-GalCer aliquots from −20 °C are single-used. Do not refreeze after diluting with media.
20. Cell expansion fold is donor-dependent. iNKT cells from different donors can be in vitro expanded for up to 3 weeks.
21. Standard BLT (human bone marrow-liver-thymus engrafted NOD/SCID/$\gamma c^{-/-}$) humanized mouse is established by co-implanting human fetal liver and thymus pieces under the renal capsule of NSG mouse together with intravenous injection of human $CD34^+$ HSCs. In our modified approach, only thymus pieces are placed under the renal capsule together with intravenous injection of engineered $CD34^+$ HSC.
22. RN-coated plate with PBS can be left for several hours in the hood.
23. Pre-stimulate $CD34^+$ cells for 12–18 h before transduction. One option is to coat the plate in the late afternoon and seed cells at around 6 pm. The next morning, add the virus for transduction.
24. The generation of human iNKT lentivirus is described in our prior publication [15]. Do not vortex, just very gently mix the concentrated virus. If necessary, adding poloxamer and PEG-2 can improve virus transduction rate [19].
25. Both fresh and frozen fetal thymus can be used for implantation. Fetal thymus should be pre-cut into 1 mm^3 cube size. Each mouse can be implanted with one to two pieces of thymus fragments.
26. Keep small portions of un-transduced and transduced $CD34^+$ cells in X-VIVO-15 media supplemented with cytokines post

virus transduction for a 72-h culture. Collect cells and perform intracellular staining of Vβ11 to detect the virus transduction efficacy.

27. Carprofen works as painkiller to relieve the pain from surgery. It can be substitute with other analgesics based on institution recommendation.
28. The viral transduction rate, the quality of human fetal thymus, and the quality of surgery will all contribute to the quality of HSC reconstitutions. BLT-iNKT mice can live around 6 months to 1 year.
29. Sorted iNKT cells are expected to expand tenfold in the first week and another tenfold in the second week.
30. Optimize antibody dilution beforehand.
31. NY-ESO-1 specific $CD8^+$ human cytotoxic T lymphocytes (CTLs, or ESO-T cells) were generated through engineering human $CD34^+$ HSCs with a TCR gene encoding a 1G4 TCR (HLA-A2- restricted, NY-ESO-1 tumor antigen-specific) and differentiating the TCR gene-engineered HSCs into CD8+ CTLs in an artificial thymic organoid (ATO) culture [17].

Acknowledgments

We thank the University of California, Los Angeles (UCLA) animal facility for providing animal support and the UCLA Virology Core for providing human blood from healthy donors. This work was supported by a Director's New Innovator Award from the NIH (DP2 CA196335, to L.Y.), a Partnering Opportunity for Translational Research Projects Award from the California Institute for Regenerative Medicine (CIRM TRAN1-08533, to L.Y.), a Stem Cell Research Award from the Concern Foundation (to L.Y.), a Research Career Development Award from the STOP CANCER Foundation (to L.Y.), and a BSCRC-RHF Research Award from the Rose Hills Research Foundation (to L.Y.). Y.-R.L. is a predoctoral fellow supported by the UCLA Whitcome Predoctoral Fellowship in Molecular Biology. We acknowledge Tasha Tsao and Emily Peng for proofreading the content.

References

1. Bendelac A, Savage PB, Teyton L (2007) The biology of NKT cells. Annu Rev Immunol 25:297–336. https://doi.org/10.1146/annurev.immunol.25.022106.141711
2. Bennstein SB (2017) Unraveling natural killer T-cells development. Front Immunol 8:1950. https://doi.org/10.3389/fimmu.2017.01950
3. Mori L, Lepore M, De Libero G (2016) The immunology of CD1- and MR1-restricted T cells. Annu Rev Immunol 34:479–510. https://doi.org/10.1146/annurev-immunol-032414-112008
4. Godfrey DI, Stankovic S, Baxter AG (2010) Raising the NKT cell family. Nat Immunol 11

(3):197–206. https://doi.org/10.1038/ni.1841

5. Godfrey DI, Berzins SP (2007) Control points in NKT-cell development. Nat Rev Immunol 7 (7):505–518. https://doi.org/10.1038/nri2116
6. Fujii SI, Shimizu K (2019) Immune networks and therapeutic targeting of iNKT cells in cancer. Trends Immunol 40(11):984–997. https://doi.org/10.1016/j.it.2019.09.008
7. Nagato K, Motohashi S, Ishibashi F, Okita K, Yamasaki K, Moriya Y, Hoshino H, Yoshida S, Hanaoka H, Fujii S, Taniguchi M, Yoshino I, Nakayama T (2012) Accumulation of activated invariant natural killer T cells in the tumor microenvironment after alpha-galactosylceramide-pulsed antigen presenting cells. J Clin Immunol 32(5):1071–1081. https://doi.org/10.1007/s10875-012-9697-9
8. Exley MA, Friedlander P, Alatrakchi N, Vriend L, Yue S, Sasada T, Zeng W, Mizukami Y, Clark J, Nemer D, LeClair K, Canning C, Daley H, Dranoff G, Giobbie-Hurder A, Hodi FS, Ritz J, Balk SP (2017) Adoptive transfer of invariant NKT cells as immunotherapy for advanced melanoma: a phase I clinical trial. Clin Cancer Res 23 (14):3510–3519. https://doi.org/10.1158/1078-0432.CCR-16-0600
9. Takami M, Ihara F, Motohashi S (2018) Clinical application of iNKT cell-mediated anti-tumor activity against lung cancer and head and neck cancer. Front Immunol 9:2021. https://doi.org/10.3389/fimmu.2018.02021
10. Bedard M, Salio M, Cerundolo V (2017) Harnessing the power of invariant natural killer T cells in cancer immunotherapy. Front Immunol 8:1829. https://doi.org/10.3389/fimmu.2017.01829
11. Torina A, Guggino G, La Manna MP, Sireci G (2018) The Janus face of NKT cell function in autoimmunity and infectious diseases. Int J Mol Sci 19(2):440. https://doi.org/10.3390/ijms19020440
12. Lundblad LKA, Gulec N, Poynter ME, DeVault VL, Dienz O, Boyson JE, Daphtary N, Aliyeva M, Ather JL, Scheuplein F, Schaub R (2017) The role of iNKT cells on the phenotypes of allergic airways in a mouse model. Pulm Pharmacol Ther 45:80–89. https://doi.org/10.1016/j.pupt.2017.05.003
13. Nair S, Dhodapkar MV (2017) Natural killer T cells in cancer immunotherapy. Front Immunol 8:1178. https://doi.org/10.3389/fimmu.2017.01178
14. de Lalla C, Rinaldi A, Montagna D, Azzimonti L, Bernardo ME, Sangalli LM, Paganoni AM, Maccario R, Di Cesare-Merlone A, Zecca M, Locatelli F, Dellabona P, Casorati G (2011) Invariant NKT cell reconstitution in pediatric leukemia patients given HLA-haploidentical stem cell transplantation defines distinct CD4+ and CD4- subset dynamics and correlates with remission state. J Immunol 186 (7):4490–4499. https://doi.org/10.4049/jimmunol.1003748
15. Smith DJ, Liu S, Ji S, Li B, McLaughlin J, Cheng D, Witte ON, Yang L (2015) Genetic engineering of hematopoietic stem cells to generate invariant natural killer T cells. Proc Natl Acad Sci U S A 112(5):1523–1528. https://doi.org/10.1073/pnas.1424877112
16. Smith DJ, Lin LJ, Moon H, Pham AT, Wang X, Liu S, Ji S, Rezek V, Shimizu S, Ruiz M, Lam J, Janzen DM, Memarzadeh S, Kohn DB, Zack JA, Kitchen SG, An DS, Yang L (2016) Propagating humanized BLT mice for the study of human immunology and immunotherapy. Stem Cells Dev 25(24):1863–1873. https://doi.org/10.1089/scd.2016.0193
17. Zhu Y, Smith DJ, Zhou Y, Li YR, Yu J, Lee D, Wang YC, Di Biase S, Wang X, Hardoy C, Ku J, Tsao T, Lin LJ, Pham AT, Moon H, McLaughlin J, Cheng D, Hollis RP, Campo-Fernandez B, Urbinati F, Wei L, Pang L, Rezek V, Berent-Maoz B, Macabali MH, Gjertson D, Wang X, Galic Z, Kitchen SG, An DS, Hu-Lieskovan S, Kaplan-Lefko PJ, De Oliveira SN, Seet CS, Larson SM, Forman SJ, Heath JR, Zack JA, Crooks GM, Radu CG, Ribas A, Kohn DB, Witte ON, Yang L (2019) Development of hematopoietic stem cell-engineered invariant natural killer T cell therapy for cancer. Cell Stem Cell 25(4):542–557. e549. https://doi.org/10.1016/j.stem.2019.08.004
18. Watarai H, Nakagawa R, Omori-Miyake M, Dashtsoodol N, Taniguchi M (2008) Methods for detection, isolation and culture of mouse and human invariant NKT cells. Nat Protoc 3 (1):70–78. https://doi.org/10.1038/nprot.2007.515
19. Masiuk KE, Zhang R, Osborne K, Hollis RP, Campo-Fernandez B, Kohn DB (2019) PGE2 and Poloxamer Synperonic F108 enhance transduction of human HSPCs with a beta-globin lentiviral vector. Mol Ther Methods Clin Dev 13:390–398. https://doi.org/10.1016/j.omtm.2019.03.005