

Two-step protocol for regeneration of immunocompetent T cells from mouse pluripotent stem cells

Tongjie Wang^{a,b}, Cui Lv^{a,b}, Fangxiao Hu^{a,b}, Lijuan Liu^{a,b}, Jinyong Wang^{a,b,c,*}

^aState Key Laboratory of Experimental Hematology, CAS Key Laboratory of Regenerative Biology, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, 510530, China; ^bGuangdong Provincial Key Laboratory of Stem Cell and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, 510530, Guangzhou, China; ^cBioisland Laboratory, 510005, Guangzhou, China

Abstract

Numerous efforts have been attempted to regenerate T cells in culture dish from pluripotent stem cells (PSCs). However, in vitro generated T cells exhibited extremely low activity and compromised immunocompetency in vivo. Here, we describe a two-step protocol for regenerating functional T cells using an inducible *Runx1-Hoxa9*-PSC (iR9-PSCs) line. The procedure mainly includes generation of induced hematopoietic progenitor cells (iHPCs) in vitro, transplantation, and development of functional induced T cells (iT) in vivo via transplantation. The entire induction process in vitro requires 21 days before iHPCs transplantation. The development of mature T cells in vivo takes 4 to 6 weeks post-transplantation. We provide a simple and reproducible approach for functional T cell regeneration from iR9-PSCs for research purpose.

Keywords: Induced hematopoietic progenitor cells, Pluripotent stem cells, T cells regeneration, Transplantation

1. INTRODUCTION

T cells play a central role in anti-tumor cell therapy. During tumor development and progression, physiological T cells in certain patients are overwhelmingly activated and functionally exhausted,^{1,2} which are not suitable for further assembling into

engineered anti-tumor T cells. Besides anti-tumor therapy, regeneration of functional T cells in vivo is urgently needed in disease-caused compromised T immune system (such as HIV infection) as well. Thus, it is necessary to explore alternative T cell source for extending T cell therapy.

So far, great efforts have been attempted in the T cell regeneration field using stem cells as starting cell materials. A hallmark is the development of the OP9-DL1/DL4 or 3D-based MS5-hDLL1/4³⁻⁵ feeder cell lines, which induce stem cells to differentiate into phenotypic T cells in vitro in the presence of essential cytokines. However, in vitro induced T cells from pluripotent stem cells (iPSCs) showed extremely low activity and immunocompetency. Another attempt to obtain functional T cells is to induce hematopoietic stem cell (HSC)-like intermediates⁶⁻⁸ and in vivo T lymphopoiesis upon transplantation. However, generating robust *bona fide* induced-HSC (iHSC) from PSCs remains inefficient.⁹ And whether this approach can generate therapeutic tumor-killing T cells is unknown. Alternatively, we have recently established a method that converted mouse pro-pre-B cells into functional mature T cells in vivo by enforcing *Hoxb5* expression.¹⁰ But the source of pro-pre-B cells is still limited. Therefore, a solid and universal approach, capable of generating immunocompetent and therapeutic T lymphopoiesis from the unlimited and gene editable PSCs, is still lacking.

In this study, we describe a detailed two-step protocol for the regeneration of functional T cells using the inducible *Runx1-Hoxa9*-PSC (iR9-PSCs) line. This method has been successfully used to induce mature T cells from either embryonic stem cells (ESCs) or iPSCs.¹¹ The induction process is initiated from EB formation. iR9-PSCs are collected and hanging in drops for embryoid bodies (EBs) formation. Next, EBs are replanted into gelatinized plates for further induction of induced hemogenic endothelial progenitors (iHECs) by culturing in specific differen-

*Address correspondence: Jinyong Wang, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, 190 Kaiyuan Avenue, Science Park, Guangzhou, 510530, China. E-mail address: wang_jinyong@gibh.ac.cn (J. Wang).

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Author contributions: T.W., C.L., and F.H. conducted all the major experiments, data analysis and wrote the manuscript. L.L. participated in multiple experiments. J.W. designed the project, wrote the manuscript and provided the final approval of the manuscript.

T.W., C.L., and F.H. contributed equally.

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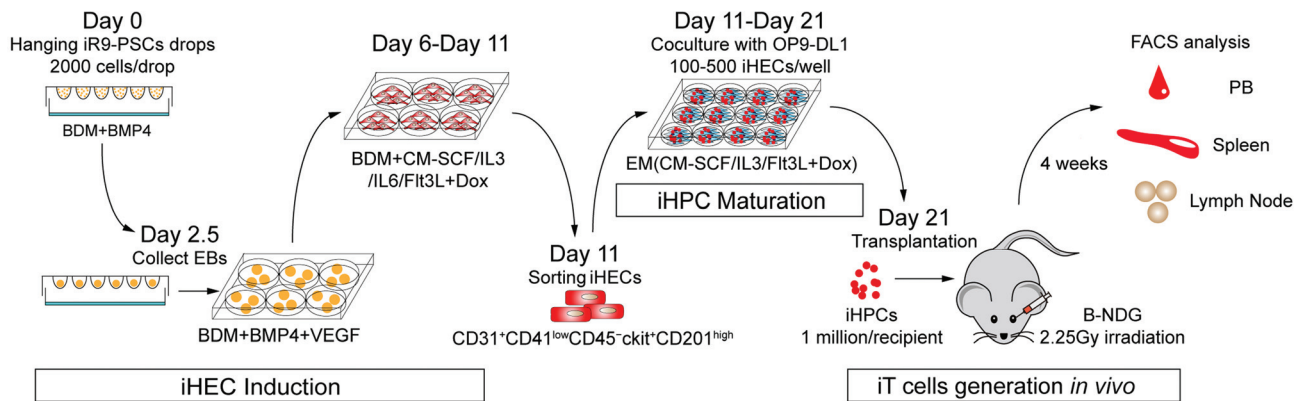


Figure 1. Schematic diagram of generating functional T lymphocytes from iR9-PSC. The differentiation process was started with EB formation and followed by iHEC induction. At day 0, iR9-PSCs were collected and hanging in drops in the basic differentiation medium (BDM: IMDM, 15% FBS, 200 μ g/mL iron-saturated transferrin, 0.45 mM MTG, 1% GlutaMAX, and 50 μ g/mL Vc) supplemented with BMP4. At day 2.5, EBs were replanted into gelatinized plates in BDM supplemented with BMP4 and VEGF. At day 6, the medium was replaced by BDM supplemented with doxycycline, 2% conditioned medium (CM) of AFT024-mIL3, AFT024-mIL6, AFT024-hFlt3L, and AFT024-mSCF cell supernatants. At day 11, 100 to 500 sorted iHECs were seeded into each OP9-DL1 covered well for hematopoietic maturation in EM medium (α -MEM, 15% DFBS, 200 μ g/mL iron-saturated transferrin, 0.45 mM MTG, 1% GlutaMAX, 50 μ g/mL Vc, 2% conditioned medium of AFT024-mIL3, AFT024-hFlt3L and AFT024-mSCF cell supernatants and 1 μ g/mL doxycycline). Finally, the iHPCs were transplanted into sublethally irradiated B-NDG recipients via orbital veins. Four weeks after transplantation, cells harvested from PB, spleen and lymph node were analyzed by flow cytometry.

tiation medium and subjected to addition of Doxycycline to turn on the expression of *Runx1-Hoxa9* at a specified time window. Then, the iHECs are sorted and cocultured with OP9-DL1 for iHPC maturation. Finally, the iHPCs are transplanted into irradiated B-NDG recipients for the final step of T cell regeneration *in vivo*. Four weeks after transplantation, mature T cells can be detected in the peripheral blood (PB), spleen and lymph node from recipients by flow cytometry analysis. We have verified that the iT cells can restore immune surveillance function in immunodeficient mice, which illustrated the iT cells had normal function.¹¹ In summary, we describe a straightforward reproducible method to preferentially generate functional T cells from mouse PSCs for research purpose.

2. EXPERIMENTAL DESIGN

We illustrate a detailed PROCEDURE for obtaining functional T cells by *in vitro* induction and *in vivo* generation, in which each part (EB formation, iHEC induction, iHPC maturation, iHPC transplantation, and analysis of iT cells) should be carefully followed to obtain optimal outcomes (Fig. 1).

2.1. Embryoid bodies formation from iR9-PSCs

EB formation from ES cells is the principal step for producing different cell lineages for further applications. An EB consists of ectodermal, mesodermal, and endodermal tissues, which recapitulate many aspects of cell differentiation during early mammalian embryogenesis and differentiate into derivatives of all the three germ layers.¹² Therefore, we take EB formation as the first step to initiate the differentiation process as well (day 0). The iR9-PSCs are collected and plated into the 0.1% gelatin-coated (Merck Millipore) well for 40 min to remove the feeder cells. Then the floating cells are collected and suspended in basic differentiation medium (BDM: IMDM, 15% FBS [Gibco], 200 μ g/mL iron-saturated transferrin, 0.1 mM β -mercaptoethanol, 1% GlutaMAX, and 50 μ g/mL ascorbic acid) supplemented with 5 ng/mL BMP4 for hanging drops. The iR9-PSCs are cultured in hanging drops (2000 PSCs/drop) for 2.5 days on the lids of 15 cm plates, in which iR9-PSCs cells are

allowed to aggregate and form spherical three-dimensional structures EB.

2.2. Induction of hemogenic endothelial progenitors

The hematopoietic cells are generated from mesodermal derived HECs during natural embryonic differentiation. Therefore, we optimize the process of inducing EB differentiation into HECs based on a previous protocol.¹³ First, the EBs are collected and replanted into gelatinized 6-well plates at day 2.5 for further endothelia induction. Meanwhile, these EBs are exposed to BMP4 (5 ng/mL) and VEGF (5 ng/mL) in BDM medium to promote generation of hemogenic endothelial (HE).¹⁴ Next, the Doxycycline (1 μ g/mL) is added to the BDM medium at day 6 to turn on the expression of *Runx1* and *Hoxa9*, which are pivotal for endothelial to hematopoietic transition (EHT). In addition, the AFT024-mSCF/mIL3/mIL6/hFlt3L cell line-conditioned medium (CM, concentration: 2%) are also used as additive for the *in vitro* induction of iHECs, which phenotypically resemble embryonic pre-HSCs. Since unwanted cell types appear during the iHEC induction process, cardiac muscle cell for example, we isolate the iHECs from the cell matrix by sorting CD31⁺CD41^{low}CD45⁻ckit⁺CD201^{high} cells¹¹ on day 11, in accordance with the pre-HSC occurrence time during the natural embryonic development.¹⁵

2.3. Maturation of induced hematopoietic progenitor cells by coculturing iHECs with OP9-DL1

The iHECs induced from EBs have the potential of differentiating into hematopoietic cells. We sort and coculture iHECs with OP9-DL1 stromal cells (100–500 iHECs cocultured with 2×10^4 OP9-DL1 in 12 well plate) for 10 days, which can facilitate T cell development *in vitro*.¹⁶ In addition, AFT024-conditioned medium of SCF, IL3, Flt3L, which is beneficial for the generation of iHPCs *in vitro*,¹⁷ is also added into the EM medium (EM medium: α -MEM, 15% DFBS [Hyclone], 200 μ g/mL iron-saturated transferrin, 0.1 mM β -mercaptoethanol, 1% GlutaMAX, 50 μ g/mL ascorbic acid, 2% CM derived from supernatants of AFT024-mIL3, AFT024-hFlt3L, and AFT024-mSCF cell culture, 1 μ g/mL doxycycline).

The Doxycycline (1 µg/mL) is constantly appended to the medium to promote iHPC maturation. During 10-day-coculture, iHPCs gradually expand and mature, which are defined as Lin⁻c-kit⁺ CD127⁺/CD135⁺/Sca1⁺. Finally, we collect the bulk iHPCs and transplant one million of the cells into sublethally irradiated (2.25 Gy) B-NDG recipients to determine their hematopoiesis ability *in vivo*.

2.4. Generation of phenotypic and functional T cells *in vivo*

To further assess the iT cell production *in vivo*, we transplant one million iR9-PSCs-derived iHPCs into irradiated (2.25 Gy) B-NDG mice (8-week-old, female, a NOD-IL2rg^{null} CD45.1 strain) via retro-orbital injection in the absence of doxycycline. The recipients are immunodeficient to avoid potential contamination of host T cells and immune rejection. These B-NDG mice are purchased from Biocytogen Jiangsu Co., Ltd (Jiangsu, China) and all mice are housed in the SPF-grade animal facility of the Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences (GIBH, CAS, China). The recipients must be conditioned by sublethal irradiation prior to transplantation. Mature iT cells can be readily detected in the lymph node, spleen, and PB of the individual recipient at 4 to 6 weeks after transplantation.

3. MATERIALS

- B-NDG (NOD-Prkdc^{Scid}IL2rg^{tm1}/Bcgen, CD45.1⁺) female mice aged 8 to 12 weeks (Biocytogen)
- DMEM/HIGH GLUCOSE (Hyclone, Cat: SH30243.01B)
- FBS (Natocor, Cat: #SFBE)
- Nonessential amino acids (NEAA, Gibco, Cat: 11140050)
- FBS (Gibco, Cat: 10100147)
- GlutamaxTM Supplement (GibcoTM, Cat: 35050-061)
- Sodium Pyruvate (Life technology, Cat: 11360-070)
- β-mercaptoethanol (MTG, Gibco, Cat: M3148-500 mL)
- PD0325901 (Selleck, Cat: S1035)
- Chir99021 (Selleck, Cat: CT99021)
- LIF (Millipore, Cat: L5181)
- α-MEM (Gibco, Cat: 12561072)
- FBS (CellMax, Cat: VS500T)
- IMDM (Gibco, Cat: 12440-053)
- Iron-saturated transferrin (Sigma, Cat: T0665)
- Ascorbic acid (Vc, Sigma, Cat: 49752)
- Gelatin (Merck Millipore, Cat: ES-006-B)
- BMP4 (R&D, Cat: 314-BP-01M)
- VEGF (Peprotech, Cat: 100-20)
- Doxycycline hyclate (Sigma, Cat: D9291)
- DFBS (Gibco, Cat: 252000-56)
- 0.25% trypsin/1 mM EDTA (Gibco, Cat: 252000-56)
- 0.05% trypsin/1 mM EDTA (Gibco, Cat: 253000-54)
- TrypLETM Express Enzyme (Gibco, Cat: 12604013)
- DPBS (Gibco, Cat: 14040133)
- BSA (EMD Millipore Calbiochem, Cat: 840224)
- Co-trimoxazole
- Penicillin-Streptomycin Solution (HyClone, SV30010-100 mL Cat: SV30010)
- Purified anti-mouse CD16/32 antibody (BioLegend Clone: 93, Cat: 14-0161-86)
- Anti-Mouse CD31 Biotin (eBioscience, Clone: 390, Cat: 13-0311-82)
- Anti-Mouse CD31 PE-Cy7 (eBioscience, Clone: 390, Cat: 25-0311-82)

- Anti-Mouse CD41 APC (eBioscience, Clone: eBioMWR30, Cat: 17-0411-82)
- Anti-Mouse CD45 Percp-Cy5.5 (eBioscience, Clone: 30-F11, Cat: 45-0451-80)
- Anti-Mouse CD117 (c-kit) APC-Cy7 (eBioscience, Clone: 2B8, Cat: 47-1171-82)
- Anti-Mouse CD201 PE (eBioscience, Clone: eBio1560, Cat: 12-2012-82)
- Streptavidin PE-Cy7 (eBioscience, Cat: 25-4317-82)
- Anti-Mouse CD2 Biotin (eBioscience, Clone: RM2-5, Cat: 13-0021-85)
- Anti-Mouse CD3 Biotin (eBioscience, Clone: 145-2C11, Cat: 13-0031-85)
- Anti-Mouse CD4 Biotin (eBioscience, Clone: RM4-5, Cat: 13-0041-85)
- Anti-Mouse CD8 Biotin (eBioscience, Clone: 53-6.7, Cat: 13-0081-85)
- Anti-Mouse CD11b Biotin (eBioscience, Clone: M1/70, Cat: 13-0112-85)
- Anti-Mouse Gr1 Biotin (eBioscience, Clone: RB6-8C5, Cat: 13-5931-85)
- Anti-Mouse Ter119 Biotin (eBioscience, Clone: TER-119, Cat: 13-5921-85)
- Anti-Mouse CD19 Biotin (eBioscience, Clone: eBio1D3, Cat: 13-0193-85)
- Anti-Mouse NK1.1 Biotin (Biolegend, Clone: PK136, Cat: 108704)
- Anti-Mouse TCRγδ Biotin (Biolegend, Clone: GL3, Cat: 118103)
- Anti-Mouse CD45 APC (Biolegend, Clone: 30-F11, Cat: 103112)
- Anti-Mouse CD117 (c-kit) PE-Cy7 (eBioscience, Clone: 2B8, Cat: 25-1171-82)
- Anti-Mouse CD135 PE (Biolegend, Clone: A2F20, Cat: 135306)
- Anti-Mouse CD127 BV421 (Biolegend, Clone: A7R34, Cat: 135027)
- Anti-Mouse Sca1 Percp-Cy5.5 (eBioscience, Clone: D7, Cat: 45-5981-82)
- Streptavidin APC-Cy7 (Biolegend, Cat: 405208)
- Anti-Mouse CD45.2 Percp-Cy5.5 (eBioscience, Clone: 104, Cat: 45-0454-82)
- Anti-Mouse CD3 PE-Cy7 (eBioscience, Clone: 145-2C11, Cat: 25-0031-82)
- Anti-Mouse CD4 PE (eBioscience, Clone: GK1.5, Cat: 12-0041-82)
- Anti-Mouse CD8 Alexa Fluor 700 (eBioscience, Clone: 53-6.7, Cat: 56-0081-80)
- Anti-Mouse TCRβ APC (eBioscience, Clone: H57-597, Cat: 17-5961-81)
- Streptavidin TCRγδ PE-Cy5 (eBioscience, Clone: eBioGL3, Cat: 15-5711-81)
- Anti-Biotin MicroBeads (Miltenyi Biotec, Cat: 130-090-485)
- DAPI (Beyotime, Cat: C1002)
- Propidium Iodide Staining Solution (PI, Biolegend, Cat: 421301)

4. EQUIPMENT

- Falcon[®] 5 mL Round Bottom Polystyrene Tube, with Snap Cap, Sterile (BD, #352054)
- Falcon[®] 5 mL Round Bottom Polystyrene Tube, with 25 mm Cell Strainer Snap Cap (BD, #352235)

- 70 mm strainer (BD, #REF 352350)
- MACS LS columns (Miltenyi Biotec)
- MidiMACS separator (Miltenyi Biotec)
- MultiStand (Miltenyi Biotec)
- FACS AriaIII sorting system (BD Biosciences) (see Equipment set up)
- BD LSR Fortessa X-20 Analysis system
- RS-2000X-Ray Irradiator (RAD SOURCE) (see Equipment set up)
- Centrifuge (Eppendorf 5810R, with swing-bucket rotor)
- 10 cm culture dish (CELLSTAR, Cat: 664160)
- 15 cm culture dish (CELLSTAR, Cat: 639160)
- 6-well culture plate (CELLSTAR, Cat: 657160)
- 12-well culture plate (CELLSTAR, Cat: 665180)
- 15 mL centrifuge tube (Corning, Cat: 430790)
- 50 mL centrifuge tube (Corning, Cat: 430828)
- 1.5 mL Microtubes (Corning, Cat:15719392)

5. REAGENT SETUP

MEF medium: DMEM/high glucose, 10% FBS (Natocor), 1% nonessential amino acids

ES medium: DMEM/high glucose, 15% FBS (Gibco), 1% NEAA, 1% GlutaMAX, 1% Sodium Pyruvate, 0.1 mM β -mercaptoethanol, 1 μ M PD0325901, 3 μ M Chir99021 and 1000 U/mL LIF

Basic differentiation medium (BDM): IMDM, 15% FBS (Gibco), 200 μ g/mL iron-saturated transferrin, 0.1 mM β -mercaptoethanol, 1% GlutaMAX, and 50 μ g/mL ascorbic acid

OP9-DL1 medium: α -MEM, 20% FBS (CellMax)

Staining buffer: 2% FBS in DPBS

MACS buffer: DPBS, 0.5% BSA, 2 mM EDTA

EM medium: α -MEM, 15% DFBS (Hyclone), 200 μ g/mL iron-saturated transferrin, 0.1 mM β -mercaptoethanol, 1% GlutaMAX, 50 μ g/mL ascorbic acid, 2% conditioned medium derived from supernatants of AFT024-mIL3, AFT024-hFlt3L and AFT024-mSCF cell culture, 1 μ g/mL doxycycline

DAPI solution: dilute the storage DAPI solution (1 mg/mL) with staining buffer to 2.5 mg/mL.

ACK Red blood cell lysis buffer: 8.024 g of NH_4Cl , 1.001 g of KHCO_3 , and 0.372 g of Na_2EDTA in 1 L H_2O .

6. EQUIPMENT SETUP

BD AriaIII Sorter used for sorting of iHEC cells. Setup the sorting system according to the BD FACSAria III User's Guide.

BD LSR Fortessa X-20 Analyzer used for cell phenotype analysis. Setup according to the BD LSR Fortessa X-20 Cell Analyzer User's Guide.

RS-2000X-Ray Irradiator used for irradiation. Setup according to manual.

7. PROCEDURE

7.1. PSCs maintenance TIMING: 7 to 10 days

! CAUTION The *Runx1-Hoxa9* cDNA has been inserted into the rTA-TRE-HygroR cassette on the Rosa26 locus of C57BL/6 mouse (CD45.2⁺) ESCs or iPSCs by homologous recombination.

1| The feeder layers should be prepared at least 12 h before thawing the PSCs.

! CAUTION PSCs are maintained on feeder layers (colchicine-treated MEF) in ES medium. Therefore, the feeder layers have to

be planted prior to ESCs thawing or passage. The feeder cells should cover the bottom of the 6-well culture dish.

2| Remove a vial of frozen iR9-PSCs from liquid nitrogen tank and put the vial into 37°C water bath until most cells are thawed.

3| Clean the vial with ethanol, and transfer the cell suspension to a 15 mL tube.

4| Add 9 mL pre-warmed ES medium to the cell suspension dropwise. Mix the suspension by shaking the tube gently.

5| Centrifuge at 250 g for 5 min at room temperature and discard the supernatant.

6| Resuspend the cells with ES medium and transfer the cells into prepared feeder dishes. Count the numbers and adjust the concentration to 10^5 cells/6-well in 2 mL ES medium. Incubate the cells in 37°C, 5% CO_2 .

7| Change the ES medium everyday. Passage the PSCs every 3–4 days before the PSC clones become dense and contact with each other.

? **Troubleshooting**

7.2. EB formation TIMING: 2.5 days

! CAUTION The growing status of PSCs is critical for achieving the long-term hematopoietic differentiation. Cells in logarithmic phase without differentiation trend are more conducive to the hematopoietic induction (Fig. 2A).

8| Gelatin-coated culture dishes should be prepared 1 h prior to suspend the EB.

9| Wash the iR9-PSCs by 1 mL DPBS, add 0.5 mL 0.05% (wt/vol) trypsin and incubate at 37°C for 3 to 4 min.

10| Add 1 to 2 mL ES medium to neutralize trypsin and suspend the cells by pipetting up and down to single-cell suspension.

11| Transfer the cell suspension to a 15 mL tube. Centrifuge at 250 g for 5 min at room temperature and discard the supernatant.

12| Resuspend the cells with ES medium and transfer the cells into gelatinized culture dish. Put the dish into incubator for 40 min to remove the feeder layers, because the MEF cells will adhere the dish faster than the PSCs.

! CAUTION Adherence time should not exceed 40 min, otherwise a lot of PSCs will be lost.

13| Collect the supernatant in the gelatinized dish, wash the dish gently by DPBS. Centrifuge at 250 g for 5 min at room temperature and discard the supernatant.

14| Wash the cells with 5 mL DPBS to get rid of the differentiation inhibitors in the ES medium. Centrifuge at 250 g for 5 min at room temperature and discard the supernatant.

! CAUTION Remove of the differentiation inhibitors in ES medium is important for the subsequent induction status.

15| Resuspend the PSCs in the BDM supplemented with 5 ng/mL BMP4. Count the cell numbers and adjust the concentration to 10^5 cells/mL.

16| Plate the iR9-PSCs supernatant at 20 μ L/drop (\approx 2000 cells/drop) on the lids of 15 cm dishes. Add some sterile distilled water into the bottom of the 15 cm dishes to prevent the evaporation of medium on the lid. Turn over the lid and close it onto the bottom of the dish for inverted culture. This point-in-time is set as day 0. Incubate the cells at 37°C, 5% CO_2 for 2.5 days.

17| Gelatinized 6-well dishes should be prepared 1 hour prior to collect the EBs.

18| At day 2.5, turn over and incline the lids, flush the EBs with DPBS. Gently collect the EBs into 50 mL tube using Pasteur pipette.

! CAUTION Do not use pipette tips to collect the EBs since it will break the EBs.

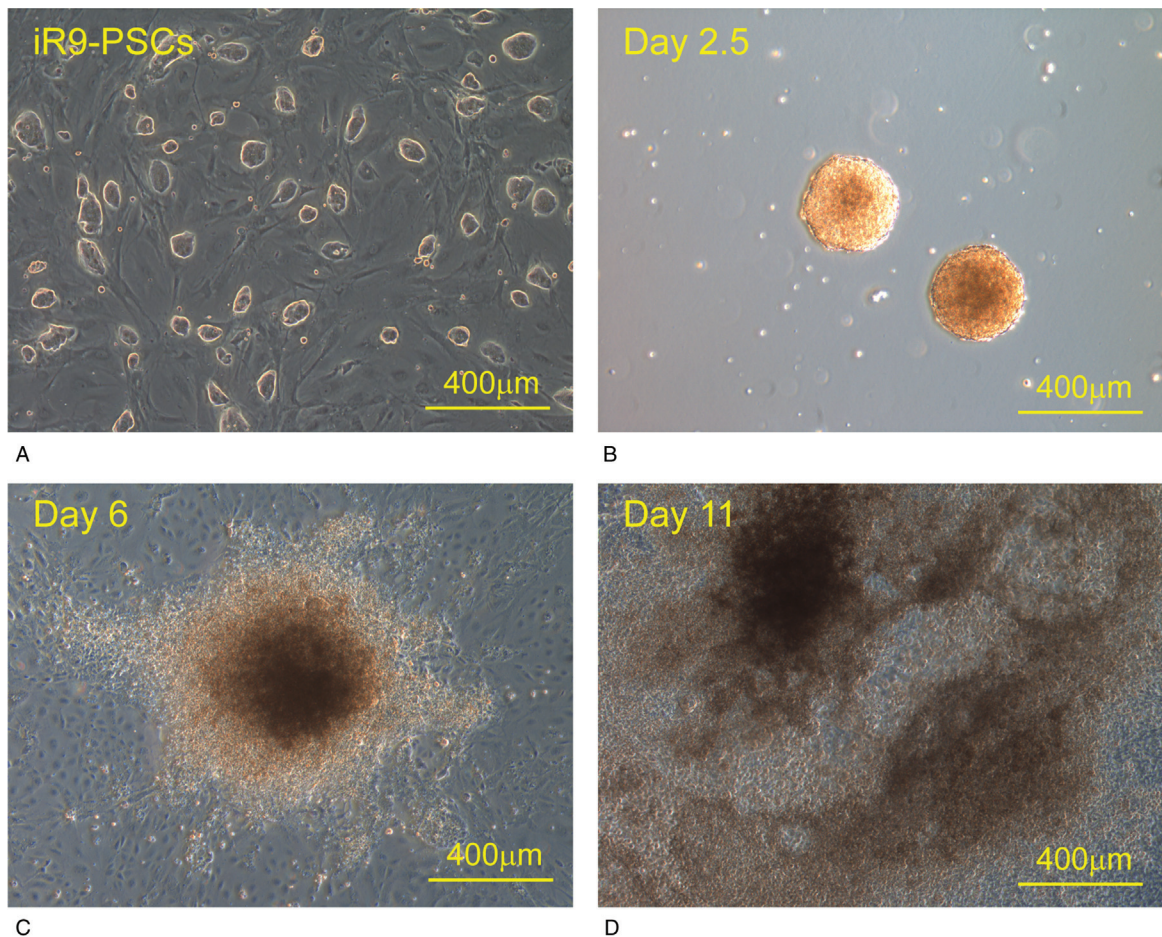


Figure 2. Morphology features of the cells during the iHECs induction from iR9-PSCs. (A) iR9-PSC clones before hanging drops for EB formation. (B) Morphology of collected EBs at day 2.5. (C) Induced endothelia cells from EBs at day 6. (D) Cell morphology of the differentiated cells at day 11.

19| Centrifuge at 90g for 5 min at room temperature and carefully discard the supernatant.

20| Resuspend the EBs with BDM medium supplemented with 5 ng/mL BMP4 and 5 ng/mL VEGF by pipetting up and down gently. EBs are replanted into gelatinized plates with a density of 30 to 40 EBs/6-well.

7.3. iHECs induction TIMING: 8 days

21| At day 4 or day 5, half change the BDM medium (supplemented with 5 ng/mL BMP4 and 5 ng/mL VEGF) carefully.

! CAUTION Due to the loosely attachment of EBs by this time, replace the medium gently and carefully to prevent the EBs floating in the medium.

22| At day 6, the medium is changed to BDM supplemented with 2% conditioned medium derived from the supernatants of AFT024-mIL3, AFT024-mIL6, AFT024-hFlt3L and AFT024-mSCF cell culture. Doxycycline (1 µg/mL, Sigma) is also added at day 6.

! CAUTION The AFT024 cells were overexpressed with the genes encoding mSCF/mIL3/mIL6/hFlt3L by retrovirus transduction in advance. Then we collect the supernatants of AFT024-mIL3/mIL6/hFlt3L/mSCF cell culture as conditioned medium.

23| Replace the medium every other day (at day 8, day 10) until iHEC sorting.

7.4. OP9-DL1 preparation TIMING: 3 days

! CAUTION The OP9-DL1 cells attach the dishes better if the dishes are coated with gelatin. Generally, a gelatinized dish should be prepared before OP9-DL1 thawing or passage.

24| Around day 7, thaw the OP9-DL1 cells for following coculture with iHPCs.

1. Remove a vial of frozen OP9-DL1 cells from liquid nitrogen tank and put the vial into 37°C water bath until most cells are thawed.

2. Clean the vial with ethanol, and transfer the cell suspension to a 15 mL tube.

3. Add 9 mL pre-warmed OP9-DL1 medium to the cell suspension dropwise. Mix the suspension by shaking the tube gently.

4. Centrifuge at 250g for 5 min at room temperature and discard the supernatant.

5. Resuspend the cells with OP9-DL1 medium and transfer the cells into prepared gelatinized dishes. Incubate the cells in 37°C, 5% CO₂.

25| Twelve hours prior to the iHPC maturation step (around day 10), collect the OP9-DL1 cells by trypsinization.

1. Wash the OP9-DL1 by 1 to 2 mL DPBS, add 1 mL 0.25% (wt/vol) trypsin and incubate at 37°C for 3 to 4 min.

2. Add 1 to 2 mL OP9-DL1 medium to neutralize trypsin and suspend the cells by pipetting up and down to single-cell suspension.
 3. Transfer the cell suspension to a 15 mL tube. Centrifuge at 250 g for 5 min at room temperature and discard the supernatant.
 4. Resuspend the cells with OP9-DL1 medium.
 - 26| Count the cell numbers of OP9-DL1 and replant the cells into gelatinized 12-well dish by 2×10^4 cells/12-well. Incubate the cells in 37°C, 5% CO₂.
- ! CAUTION** Excess OP9-DL1 cells seeded in the 12-well will result in drift of OP9-DL1 feeder layers due to the excessive cell density at the later stage of iHPC maturation.

7.5. iHEC sorting TIMING: 10 to 12 h

- 27| Collect the medium in the culture dish and filter through a 70 µm strainer into a 50 mL tube, wash the adherent cells by DPBS and filter the DPBS into the 50 mL tube as well. Add 0.5 mL TrypLE to each well and incubate at 37°C for 10 to 15 min.
 - 28| Add 1 mL DPBS and suspend the cells by pipetting up and down to single-cell suspension.
 - 29| Filter the cells through a 70 µm strainer into the 50 mL tube.
 - 30| Centrifuge the cells for 5 min at 500g at 4°C. Remove the supernatant carefully.
 - 31| Resuspend the cells with staining buffer. Count the cell numbers and adjust the concentration to 10^8 cells/mL.
 - 32| Add 0.5 µL anti-mouse CD16/32 antibody per 10^7 cells. Incubate the cells on ice for 15 min.
 - 33| Add 0.5 µL Biotin anti-Mouse CD31 antibody per 10^7 cells. Incubate the cells on ice for 20 to 30 min.
 - 34| Wash the cells with 5 volume of staining buffer. Centrifuge for 5 min at 500g and 4°C. Discard the supernatant.
 - 35| Adjust the cell concentration to 2.5×10^8 /mL with MACS buffer. Add 40 µL anti-biotin microbeads per 10^8 cells and incubate for 30 min at 4°C in dark.
 - 36| Wash once with 5 volume of MACS buffer. Centrifuge the cells at 500g, 4°C for 5 min. Resuspend the cells with MACS buffer.
 - 37| Pass the cell suspension through a 25 µm strainer.
 - 38| Place a LS column on MidiMACS separator assemblies with Multistand.
 - 39| Add 4 mL MACS buffer to pre-wet the LS column.
 - 40| Place a 15 mL tube to collect the CD31 negative cells.
 - 41| Add the microbeads incubated cells suspension, let the cells pass through the LS column.
 - 42| Wash the LS column with 4 mL MACS buffer twice.
 - 43| Pipette 4 mL MACS buffer onto the LS column. Immediately flush out fraction with the magnetically labeled cells into another 15 mL tube by firmly applying the plunger supplied with the column. Centrifuge the cells for 5 min at 500g and 4°C.
- ! CAUTION** Enrichment of CD31 positive cells can save time of sorting iHECs. If the collected cells are not too much, we do not have to enrich the CD31⁺ cells, just skip step 33 to step 43.
- 44| Resuspend the cells with staining buffer and transfer them into Falcon[®] 5 mL Round Bottom Polystyrene Tube.
- ! CAUTION** Cell concentration would be around 10^8 /mL.
- 45| Suspend the cells with appropriate volume of staining buffer and stain them with antibodies including anti-Mouse CD31 PE-Cy7, anti-Mouse CD41 APC, anti-Mouse CD45 Percp-Cy5.5, anti-Mouse CD117 (c-kit) APC-Cy7, anti-Mouse CD201 PE (1:200), and Streptavidin PE-Cy7 (1:400) for 20–30 min on ice.
 - 46| Wash with 5 volumes of staining buffer, and centrifuge for 5 min at 500g and 4°C.

- 47| Resuspend the cells with DAPI solution.
- ! CAUTION** Keep the cells on ice and in dark until sorting.
- 48| Sort the iHEC cells with an BD FACS Aria III sorter instrument.
1. Prepare a 1.5 mL tube for collecting iHECs filled with 0.5 mL EM medium (add $2 \times$ Penicillin-Streptomycin to avoid contamination).
 2. Check the purity and viability of sorting cells.
 3. Spin down the collected iHECs by centrifuge for 5 min at 500g and 4°C.
 4. Resuspend the cells with EM medium and count the cell number.
- ? Troubleshooting**

7.6. iHPC maturation TIMING: 10 days

- 49| Seed 100 to 500 sorted iHECs into each OP9-DL1 covered well. Incubate the cells in 37°C, 5% CO₂.
 - 50| Half replace the EM medium carefully every 2 days until iHPCs transplantation.
- ? Troubleshooting**
- 51| After 10-day coculture, collect 1 million bulk cells and filter into Falcon[®] 5 mL Round Bottom Polystyrene Tube. Centrifuge the cells for 5 min at 500g and 4°C.
 - 52| Resuspend the cells with 200 µL staining buffer. Add 1 µL purified anti-mouse CD16/32 antibody to each sample. Incubate the samples on ice for 15 min.
 - 53| Suspend the cells with appropriate volume of staining buffer, and stain with the antibodies of anti-mouse CD2/CD3/CD4/CD8/CD11b/Gr1/Ter119/CD19/NK1.1/ TCRγδ Biotin (1:200) for 20 to 30 min on ice in dark.
 - 54| Add $5 \times$ volume staining buffer into the staining tubes and centrifuge at 4°C, 500g for 5 min and then discard the supernatant.
 - 55| Add the related antibody (anti-mouse CD45 APC, anti-mouse CD117 PE-Cy7, anti-mouse CD135 PE, anti-mouse CD127 BV421, anti-mouse Sca1 Percp-Cy5.5, streptavidin APC-Cy7) mix buffer into the related samples. Incubate on ice in dark for 20 to 30 min.
 - 56| Add 400 µL PI solution (dilution: 1:50) to each sample and resuspend the cells. Place the stained sample on ice.
 - 57| Flow cytometry analysis on BD LSR Fortessa X-20 analyzer.

7.7. Transplantation of iHPC TIMING: 6 h

- 58| At day 21, count the cell number of iHPCs, estimate the number of recipients.
 - 59| Irradiate the recipient mice (8–10-week-old B-NDG mice) with 2.25 Gy (1.0 Gy/min) at least 4 h before transplantation.
- ! CAUTION** Feed the recipients with co-trimoxazole from several days (3–7 days) before transplantation to two weeks after transplantation.
- 60| Collect the suspending cells and filter the cells through a 70 µm strainer into a 50 mL tube.
 - 61| Wash the adherent cells with DPBS and collect the DPBS into the above 50 mL tube.
 - 62| Add 0.5 mL TrypLE to each well and incubate at 37°C for 10 to 15 min.
 - 63| Add 1 mL EM medium to neutralize TrypLE and suspend the cells by pipetting up and down to single-cell suspension.
 - 64| Filter the cells through a 70 µm strainer into the above 50 mL tube.
 - 65| Centrifuge the cells for 5 min at 500g at 4°C. Remove the supernatant carefully.

66| Resuspend the cells with staining buffer. Count the cell number and adjust the cell concentration to $3 \times 10^6/\text{mL}$.

67| Anesthesia the irradiated recipients by breathing with isoflurane.

68| Transplant the 350 μL cell suspension into sublethally irradiated recipients via retro-orbital veins.

! CAUTION Transplantation should be conducted at least 4 h after irradiation.

? Troubleshooting

7.8. Flow cytometry analysis of the iT cells TIMING: 5 h

! CAUTION The immune organs of the recipients transplanted with the iHPCs should be analyzed at least 4 weeks after transplantation.

Alexa Fluor 700, anti-mouse TCR β APC, anti-mouse TCR $\gamma\delta$ PE-Cy5) mix buffer into the related samples. Incubate on ice in dark for 20 to 30 min.

78| Add 5 \times volume staining buffer into the staining tubes and centrifuge at 4°C, 500g for 5 min and then discard the supernatant.

79| Add 800 μL DAPI solution to each sample and resuspend the cells. Place the stained sample on ice.

80| Flow cytometry analysis on BD LSR Fortessa X-20 analyzer.

? Troubleshooting

8. TROUBLESHOOTING

Step	Problem	Possible	Solution
7	Bad status of PSCs	1. Inefficient reagents used in ES medium. 2. Inadequate quantity of feeder cells. 3. Dense colonies of PSCs.	1. Change the reagents in the ES medium. 2. Seed sufficient quantity of feeder. 3. Passage the PSCs in time.
48	Few iHECs	1. Bad status of initiated PSCs. 2. Inaccurate cell number of PSCs in hanging EB drops. 3. Incorrect medium for iHEC induction. 4. High mortality of cells in sorting sample.	1. Start the differentiation after adjustment of the PSCs status. 2. Count the PSCs precisely before hanging the EB drops. 3. Prepare the medium according to this protocol carefully. 4. Collect the sorting cells gently as soon as possible. Shorten the operation time at room temperature. Always put the sample on ice.
50	OP9-DL1 cells floated in the coculture process	1. Excess OP9-DL1 cells seeded in each 12-well. 2. Overgrowth of iHPCs.	1. Transfer the iHPCs to new OP9-DL1 feeder cells once they are floating. 2. Divided the filled iHPCs into several OP9-DL1 seeded wells.
80	Transplantation failure	1. Insufficient dose of donor iHPCs. 2. Wrong irradiation dose. 3. Inefficient cytokines used in differentiation. 4. High mortality of iHPCs.	1. Transplant enough dose of iHPCs into each recipient. 2. Measure the weight of mouse before irradiation (2.25 Gy/20–25 g). 3. Change the cytokines used in the induction process in vitro. 4. Prepare the well-grown OP9-DL1 cells before coculture. Change the OP9-DL1 feeder cells once they are floated. Divided the filled iHPCs into several OP9-DL1 seeded wells. Collect the iHPCs gently and carefully.

69| Anesthesia the irradiated recipients by breathing with isoflurane.

70| Collect 500 μL PB into 1 mL anticoagulation tube by ophthalmic vein blood sampling.

71| Sacrifice the recipients and dissociate the immune organs. The immune organs include spleen and lymph node.

! CAUTION The B-NDG mice are severely immunodeficient mice whose thymus are abnormal.

72| Grind the immune organs in the staining buffer using a syringe plunger. Pass the cell suspension through a 70 μm strainer into the 15 mL centrifuge tubes. Centrifuge the cell suspension at 4°C, 500g for 5 min and then discard the supernatant.

73| Add 1 mL ACK red blood cell lysis buffer to the PB and spleen cells to lyse the red cells. For the PB, the lysis time is 20 min, and for the spleen cells, the lysis time is 3 min. Centrifuge the cell suspension at 4°C, 500g for 5 min and then discard the supernatant.

74| Add 4 mL staining buffer and resuspend the cells. Centrifuge at 4°C, 500g for 5 min and then discard the supernatant. Add 500 μL staining buffer and resuspend the cells and count the cells.

75| Take 5 million cells of each sample. Adjust the cell suspension volume to 200 μL for staining.

76| Add 1 μL purified anti-mouse CD16/32 antibody to each sample. Incubate the samples on ice for 15 min.

77| Add the related antibody (anti-Mouse CD45.2 Percp-Cy5.5, anti-mouse CD3 PE-Cy7, anti-mouse CD4 PE, anti-mouse CD8

9. ANTICIPATED RESULTS

In this protocol, iR9-PSCs were induced to generate hematopoietic progenitors in vitro, which gave rise to functional T cells in vivo after transplantation. We initiated the induction of iR9-PSCs by culture in hanging drops without Doxycycline's existence. After 2.5 days, the EBs formed in each drop. The EBs were collected and suspended in differentiation medium for induction (Fig. 2B). At day 6, the PSCs tended to differentiate into mesoderm derived endothelial cells (Fig. 2C). Then, the EHT was facilitated by turning on the expression of Runx1-Hoxa9 and addition of SCF, IL3, IL6, and Flt3L conditioned medium. The iHECs would gradually appear in the next few days (Fig. 2D). At day 11, the iHECs were isolated by sorting CD31⁺CD41^{low}CD45⁻ckit⁺CD201^{high} cells (Fig. 3) and cocultured with OP9-DL1 for 10 days for iHPC maturation (Fig. 4). Generally, 500 iHECs could generate 2 to 3 million bulk cells after 10 days coculture, including the phenotypic iHPCs which were defined as Lin⁻c-kit⁺ CD127⁺/CD135⁺/Sca1⁺ (Fig. 5). At day 21, one million iHPCs were transplanted into irradiated B-NDG recipients via retro-orbital vein injection. Four weeks after transplantation, abundant mature iT cells would occur in the PB (5–20%), spleen and lymph node of the recipients. Mature CD45P and CD8SP iT cells would be detected in the PB, spleen and lymph node of iT-B-NDG mice, the majority of which were TCR β positive (Fig. 6).

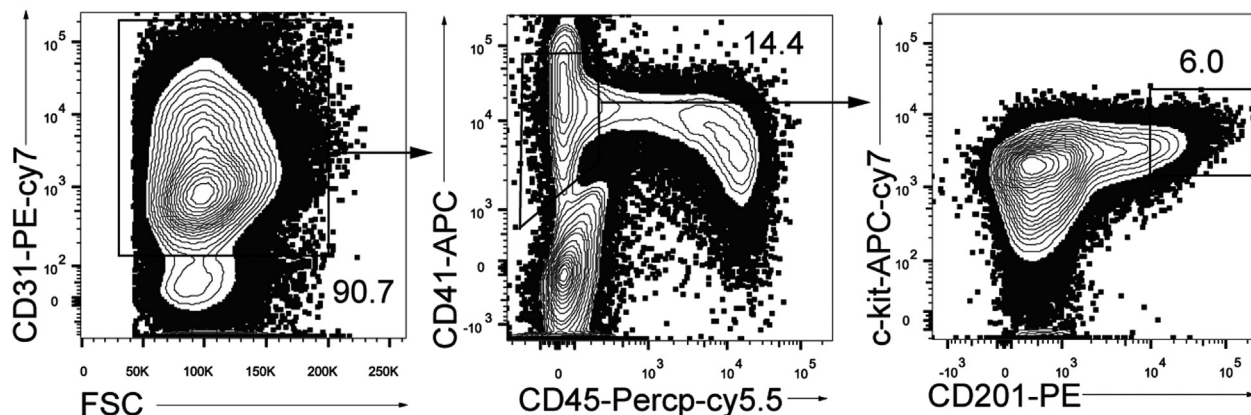


Figure 3. Gating strategy of sorting iHECs by flow cytometry at day 11. The differentiated cells were collected and enriched by incubation with biotin-conjugated anti-CD31 antibody. Then, the iHECs were sorted from the CD31⁺ enriched cells using the CD31⁺CD41^{low}CD45⁻c-kit⁺CD201^{high} combined markers.

10. DISCUSSION

This two-step protocol can efficiently generate functional T cells from mouse PSCs by inducible expression of *Runx1-Hoxa9*. We had verified the function of iT cells by skin graft rejection assay, CAR-T and TCR-T therapy.¹¹ First, we transferred the iT cells isolated from the iT-B-NDG spleen into Rag1^{-/-} recipients (iT-Rag1^{-/-} mice) to evaluate the function of iT cells derived from iR9-PSCs (C57BL/6) by skin graft rejection. Four days after

the adoptive iT cells transfer, we transplanted allogeneic skin from BALB/c mice into the iT-Rag1^{-/-} mice. The allogeneic skin was rapidly rejected by iT-Rag1^{-/-} mice at around day 9 after transplantation, as indicated by bulged, ulcerative and necrotic lesions at the graft sites. And the iT cells rejected the secondary allogeneic skin grafts again. It indicated the iT cells reject allogeneic skin and form memory response in vivo. Besides the skin graft rejection, the functions of the iT cells generated by this

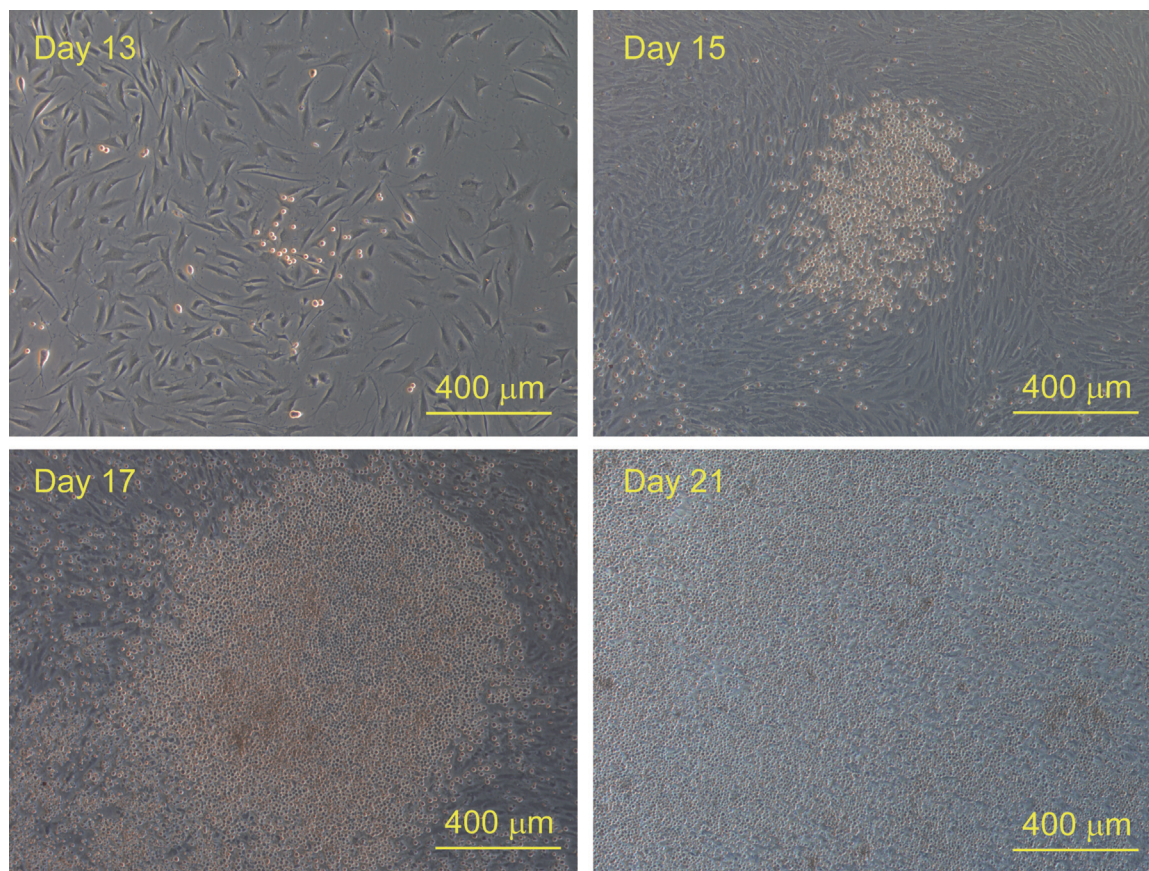


Figure 4. Cell morphology during iHPC maturation. At day 11, 500 sorted iHECs were seeded into OP9-DL1 covered dish. The iHPC gradually matured and expanded during the 10-day coculture. Representative pictures of iHPC at day 13, day 15, day 17, and day 21 were shown.

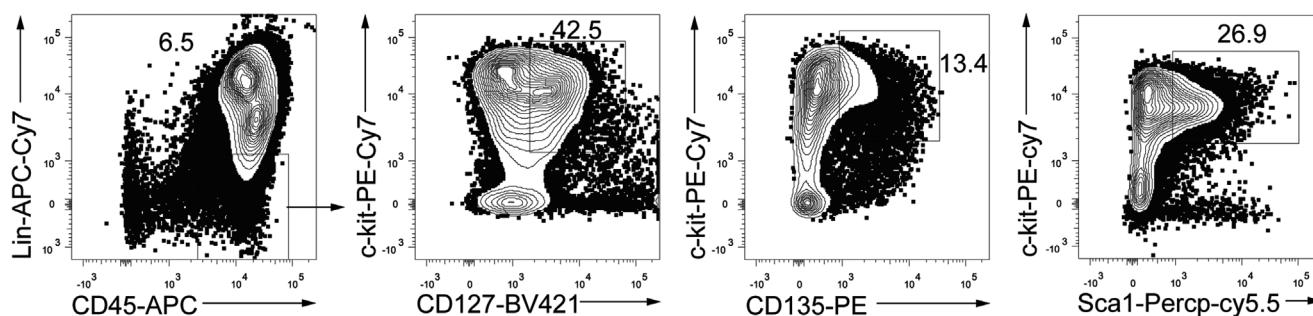


Figure 5. Immuno-phenotypes of iHPCs after 10-day maturation (at day 21). Lin was defined as $CD2^{-}CD3^{-}CD4^{-}CD8^{-}CD11b^{-}Gr1^{-}Ter119^{-}CD19^{-}NK1.1^{-}TCR\gamma\delta^{-}$. iHPCs were defined as $Lin^{-}c-kit^{+}CD127^{+}/CD135^{+}/Sca1^{+}$.

two-step protocol have been extensively assessed using CAR-iT cell therapy. The iT cells were obtained from the iT-B-NDG spleen and transduced with CD19-CAR element by retroviral infection in vitro. The CD19-CAR-iT cells were then transplanted

into the luciferase-reporting B cell lymphoma (Ka539-luciferase) mouse model. The CD19-CAR-iT cells cannot only eliminate the tumor cells which assessed by in vivo live imaging, but also can extend the life span of the treated tumor bearing mice

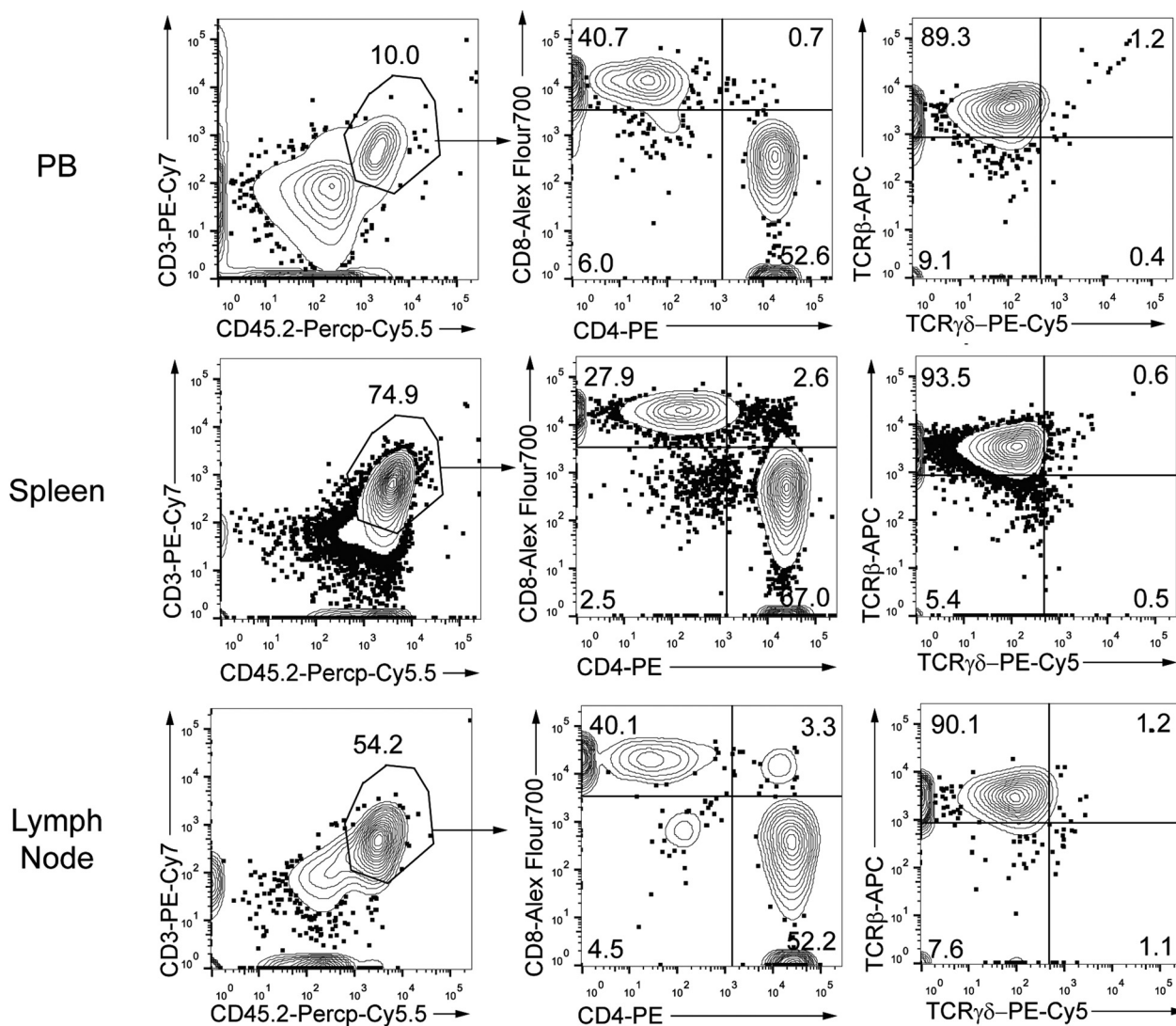


Figure 6. Flow cytometry analysis of iT lymphocytes in recipients transplanted with iR9-PSCs derived iHPCs. One million iHPCs were transplanted into sublethally irradiated B-NDG mice. Seven weeks after transplantation, the single nucleated blood cells harvested from PB, spleen, and lymph node were analyzed by flow cytometry. Representative plots were shown.

(unpublished data). Moreover, we introduced tumor antigen-specific TCR (MHC-I-restricted OVA TCR, OT1) into iR9-PSCs and further assessed the anti-tumor activity of the derived OT1 iT cells. We reconstituted the OT1-iT lymphopoiesis in the Rag1^{-/-} mice by transplanted the OT1-iHPCs into irradiated Rag1^{-/-} mice. Then, we engrafted E.G7-OVA tumor cells into the groin of the OT1-iT-reconstituted Rag1^{-/-} mice (OT1-iT-Rag1^{-/-} mice) by subcutaneous injection. Tumor growth kinetics demonstrated that the E.G7-OVA tumors were dramatically inhibited in the OT1-iT-Rag1^{-/-} mice. In summary, these results suggest that the iT cells generated by this two-step protocol have normal functions.

The *Runx1* and *Hoxa9* are both oncogenes which can induce tumorigenesis. Therefore, we only turn on the expression of these two genes during the induction process in vitro, but stop their expression once the iHPCs are transplanted in vivo. We have maintained the B-NDG recipients transplanted with the iHPCs for more than 1 year, we did not observe any tumor cells appeared in the recipients until now. We will keep an eye on the B-NDG recipients to confirm whether the transplanted iHPCs are tumorigenic.

By a consecutive process of EB formation, iHEC induction, iHPC maturation, and transplantation, we can generate abundant functional mature T cells in the recipients. Therefore, we have developed a reliable and efficient method to regenerate T cells from PSCs. Using this protocol, large amounts of functional T cells can be obtained in a period of 2-month time. The functions and translational potential of the iT cells generated by this two-step protocol have been extensively assessed.¹¹

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