Feeder-free differentiation and expansion for T cells from induced pluripotent stem cells

Shoichi Iriguchi  
Kyoto University

Yutaka Yasui  
Kyoto University

Yohei Kawai  
Kyoto University

Suguru Arima  
Takeda Pharmaceutical Company

Mihoko Kunitomo  
Takeda Pharmaceutical Company

Takayuki Sato  
Takeda Pharmaceutical Company

Tatsuki Ueda  
Kyoto University

Atsutaka Minagawa  
Kyoto University

Yuta Mishima  
Kyoto University

Nariaki Yanagawa  
Kyoto University

Yuji Baba  
Takeda Pharmaceutical Company

Yasuyuki Miyake  
Kyoto University

Kazuhide Nakayama  
Takeda Pharmaceutical Company

Maiko Takiguchi  
Takeda Pharmaceutical Company

Tokuyuki Shinohara  
Takeda Pharmaceutical Company

Tetsuya Nakatsura  
National Cancer Center

Masaki Yasukawa
Method Article

Keywords: T cell immunotherapy, iPS Cells, Differentiation,

DOI: https://doi.org/10.21203/rs.3.pex-1271/v1

License: ☕️ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract
Clinical efficacy demonstrated by chimeric antigen receptor T cell therapy call for further development that could broaden their applicability. One such direction is to develop alternate T-cell sources and T cells differentiated from pluripotent stem cells may be an ideal candidate.

The present protocol provides a feeder-free and scalable method to generate T lymphocytes from induced pluripotent stem cells.

Introduction
Recent clinical efficacy obtained with chimeric antigen receptor T cell therapy indicated that T cells could be used to treat a wide range of diseases. The development of “off-the-shelf” T cells will further expand their applications. T cells derived from induced pluripotent stem cells (iPSCs) may became an ideal cell source for this purpose\(^1\). Although our group and others have demonstrated therapeutic efficacy of iPSC-derived T cells, they are differentiated on murine stromal cell lines and are proliferated on irradiated human peripheral blood mononuclear cells, raising safety concerns and scalable issues\(^2-7\). To overcome these issues, we have developed a feeder-free differentiation protocol to generate and proliferate T cells from induced pluripotent stem cells.

Reagents
- StemFit AK03N (Ajinomoto)
- iMatrix 511 (Matrixome)
- StemPro-34 SFM (ThermoFischer)
- Insulin-Transferrin-Selenium Solution (ITS), 100x (TermoFischer)
- Ascorbic acid 2-phosphate (SIGMA)
- l-glutamine, penicillin, streptomycin (SIGMA)
- GlutaMAX (Thermo Fischer)
- 2-Mercaptoethanol (TermoFischer)
- Sodium bicarbonate (Nacalai)
- D-PBS (Ca\(^{2+}\) and Mg\(^{2+}\) free; Nacalai)
- EDTA (0.5 M, pH 8.0; Nacalai)
- Monothioglycerol (MTG, Nacalai)
• FBS, defined (HyClone etc...)
• TrypLE Select (ThermoFischer)
• Human basic FGF (Wako)
• Human BMP4 (R&D systems)
• Human Flt3 ligand (L) (Peprotech)
• Human SCF (R&D systems)
• Human VEGF (R&D systems)
• Human IL-7 (peprotech)
• Human IL-15 (PeproTech)
• Human IL-21 (PeproTech)
• Human IL-12 (Merck)
• Human IL-18 (MBL)
• Human TL-1A (PeproTech)
• Y-27632 (Tociris)
• CHIR99021 (Tocris)
• SB431542 (FujiFilm Wako)
• Trypan blue solution (0.4%; Nacalai)
• BSA fraction V (SIGMA)
• PE/Cy7 Anti-human CD34 (4H11 Abcam ab155358)
• APC Anti-human CD43 (1G10 BD Pharmingen 560198)
• FITC Anti-human CD235a (GA-R2 (HIR2) BD Pharmingen 559943)
• APC/eF780 Anti-human CD14 (61D3 eBioscience 470149-42)
• Purified Anti-human CD3 (clone: OKT3, eBioscience)
Reagent setup

- **α-MEM basal medium** Prepare according to the manufacturer’s instruction. Divide into 210-ml aliquots and store at 4 °C for up to 2 weeks.

- **FACS buffer** FACS buffer contains 2% (vol/vol) FBS in D-PBS. Store at 4 °C for up to 6 months.

- **MTG solution** Add 87 μl of MTG to 10 ml of endotoxin-free reagent-grade distilled water. Mix well and divide into 500-μl aliquots. Store for up to 6 months at -20°C.

- **Ascorbic acid solution** Add 50 mg of ascorbic acid to 1 ml of endotoxin-free reagent-grade distilled water. Dissolve completely, divide into 100-μl aliquots and store for up to 6 months at -20°C.

- **Reconstitution of cytokines** Reconstitute cytokines according to the product information provided by manufacturer.

- **Fc-DLL4 (Sino Biological Inc.)** Reconstitute at 250 µg/mL in sterile water and store at -20°C as stock solution.

- **Retronectin® (TAKARA BIO Inc.)** Dilute the Retronectin® with sterile water at 10 µg/mL and store at -20°C as stock/working solution.

Equipment

- Cell strainer (70 μm; ThermoFischer Scientific)

- Tissue culture six-well plate (polystyrene flat bottom; TPP)

- 6-well ultra-low attachment plate (Corning)

- 48-well plate (Corning)

- Polystyrene round-bottom tube (5 ml, with 35-μm cell strainer cap; BD Biosciences)

- Polypropylene conical tube (15, 50 ml)

- 1 ml syringe (Terumo)

- 21-G needle (Terumo)

- Microcentrifuge

- Sterile biosafety cabinet

- CO₂ incubator (37 °C/5%; Thermo Scientific)
• Flow cytometer (Becton Dickinson)
• Microcentrifuge tube (1.5 ml)

**Procedure**

**Hematopoietic differentiation**

**Day 0:**

To initiate differentiation, prepare a single-cell suspension of iPSCs expanded for 6–7 days on iMatrix-511 in StemFit AK03N using 0.5× TryPLE select

Resuspend a total of 3–6×10^5 cells in StemFit AK03N supplemented with 10 µM Y-27632 and 10 µM CHIR99021 and seed them in a well of 6-well ultra-low attachment plates

**Day 1:**

Harvest the EBs, settle them down to the bottom of the tube, remove the supernatant, and resuspend them in 2 ml StemPro-34 supplemented with 10 ng/ml penicillin/streptomycin, 2 mM Glutamax, 50 µg/ml ascorbic acid, 4×10^-4 M monothioglycerol and 1× Insulin-Transferrin-Selenium solution (referred to as EB basal medium), 50 ng/ml BMP-4, 50 ng/ml rhVEGF, and 50 ng/ml bFGF per well.

**Day 2:**

Add 6 µM SB431542 to each well.

**Day 4:**

Harvest the differentiating EBs as described in day 1 and resuspend them in 2 ml EB basal medium supplemented with 50 ng/ml rhVEGF, 50 ng/ml rhbFGF and 50 ng/ml rhSCF per well.

**Day 7:**

Harvest the differentiating EBs and resuspend them in 2 ml EB basal medium supplemented with 50 µg/ml rhVEGF, 50 ng/ml rhbFGF, 50 ng/ml rhSCF, 30 ng/ml rhTPO, and 10 ng/ml FLT3L per well.
From days 9-14:

Harvest the differentiating cultures and replace the spent medium with fresh day 7 medium for every 2–3 days.

Cultures should be maintained in a 5% CO₂/5% O₂/90% N₂ environment for the first 7 days and in a 5% CO₂ environment from day 7.

T-cell differentiation

Days 13, 20, 27:

Dilute rhDL4/Fc chimera protein solution (10 µg/ml) with an equal volume of Retronectin (10 µg/m) and add 150 µl of the solution to each well of 48-well plates.

Incubate the plate overnight at 4 °C. Remove the coating solution just before adding T-cell differentiation medium.

Day 14:

For iHPC seeding, harvest day 14 EBs and dissociated them into single cell by TryPLE Select treatment, followed by passing through a 21-G needle 7 times. FACS-sort a total of 2000 CD235a⁻/CD14⁻/CD34⁺/CD43⁺ cells into wells of a DL4-coated plate having T-cell differentiation medium composed of αMEM (Thermo Fisher Scientific) supplemented with 15% FBS (Corning), 100× ITS-G (1×), 55 µM 2-Mercaptoethanol, 50 µg/ml ascorbic acid, 2 mM Glutamax, 50 ng/ml rhSCF, 100 ng/ml rhTPO, 50 ng/ml rhIL-7, 50 ng/ml FLT3L, 30 nM rhSDF-1α (PeproTech), and 15 µM SB203580.

Days 15, 17, 19:

Change a major portion of the medium (80%) every other day.

Day 21:

Transfer the differentiating cells to a new DL4-coated plate
Days 23, 25, 27:
Change a major portion of the medium (80%) every other day.

Day 28:
Transfer a total of $1-2 \times 10^5$ cells/well to a new DL4-coated plate.

Days 30, 32, 34:
Change a major portion of the medium (80%) every other day.

**T cell maturation**

Day 35:
Stimulate the DL4 cells with a monoclonal antibody to CD3 at the concentration of 500 ng/ml in maturation medium composed of αMEM, 15% FBS, 100× ITS-G (1×), 50 µg/ml ascorbic acid, 100× PSG (1×, Sigma), 10 ng/ml rhIL-7, 10 ng/ml rhIL-2, and 10 nM dexamethasone. Seed $5 \times 10^5$ cells/well of 48-well plate with 1 ml of the maturation medium.

Day 38:
Collect the cells and resuspend them in maturation medium without OKT3 and incubate for 4 days.

**T-cell proliferation**

Day 41:
On the day before T-cell activation, coat 48-well plates with CD3/Retronectin solution composed of 3.0 µg/ml anti-human CD3 and 150 µg/ml Retronectin at 4 °C overnight.

Day 42:
Resuspend a total of $4 \times 10^5$ iT-cells/ml in T-cell activation medium composed of αMEM supplemented with 15% FBS, 100× ITS-G (1×), 50 µg/ml ascorbic acid, 10 ng/ml rhIL-7, 10 ng/ml rhIL-15, 20 ng/ml rhIL-21, 50 ng/ml rhIL-12, 50 ng/ml rhIL-18, 50 ng/ml rhTL-1A, and 10 µM Z-VAD. Seed the cell suspension to a well of a 48-well plate.

Day 45:

Collect the cultures and resuspend them in proliferation medium composed of αMEM, 15% FBS, 100× ITS-G (1×), 50 µg/ml ascorbic acid, 10 ng/ml rhIL-7, and 10 ng/ml rhIL-15.

Days 47, 49, 51, 53, 55

Change approximately 80% spent medium every 2–3 days, with re-culturing to new wells or larger-culture vessels as needed.

Troubleshooting

Time Taken

Anticipated Results

References

1. Themeli, M., Rivière, I. & Sadelain, M. New Cell Sources for T Cell Engineering and Adoptive Immunotherapy. *Cell Stem Cell* 16, 357–366 (2015).

2. Nishimura, T. et al. Generation of rejuvenated antigen-specific T cells by reprogramming to pluripotency and redifferentiation. *Cell Stem Cell* 12, 114–126 (2013).

3. Wakao, H. et al. Expansion of functional human mucosal-associated invariant T cells via reprogramming to pluripotency and redifferentiation. *Cell Stem Cell* 12, 546–558 (2013).

4. Vizcardo, R. et al. Regeneration of human tumor antigen-specific T cells from iPSCs derived from mature CD8(+) T cells. *Cell Stem Cell* 12, 31–36 (2013).

5. Themeli, M. et al. Generation of tumor-targeted human T lymphocytes from induced pluripotent stem cells for cancer therapy. *Nat. Biotechnol.* 31, 928–933 (2013).
6. Maeda, T. et al. Regeneration of CD8αβ T Cells from T-cell-Derived iPSC Imparts Potent Tumor Antigen-Specific Cytotoxicity. *Cancer Research* **76**, 6839–6850 (2016).

7. Minagawa, A. et al. Enhancing T Cell Receptor Stability in Rejuvenated iPSC-Derived T Cells Improves Their Use in Cancer Immunotherapy. *Cell Stem Cell* **23**, 850–858.e4 (2018).

**Acknowledgements**

This work was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (23591413, 15H04655, 15J05263, 26293357, and 18K16085), Japan Agency for Medical Research and Development (Project for Development of Innovative Research on Cancer Therapeutics, Practical Research for Innovative Cancer Control, and Core Center for iPS Cell Research), the Takeda-CiRA collaboration program, and the collaborative research grant of Thyas Co., Ltd.