Research Square

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

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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℃ as stock solution.

- **Retronectin® (TAKARA BIO Inc.**) Dilute the Retronectin® with sterile water at 10 µg/mL and store at -20℃ 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
- CO_2 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% CO_2/5% O_2/90% N_2 environment for the first 7 days and in a 5% CO_2 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 1–2×10^5 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 5x10^5 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 4×10^5 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 aMEM, 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

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