Chapter 6

In Vitro Generation of Antigen-Specific T Cells from Induced Pluripotent Stem Cells of Antigen-Specific T Cell Origin

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Abstract

Induced pluripotent stem (iPS) cells derived from T lymphocyte (T-iPS cells) preserve the T cell receptor (TCR) α and β gene rearrangements identical to the original T cell clone. Re-differentiated CD8 single positive αβ T cells from the T-iPS cells exhibited antigen-specific cytotoxicity, improved proliferative response, and elongation of telomere indicating rejuvenation of antigen specific T cell immunity in vitro. To regenerate antigen specific cytotoxic T lymphocytes (CTL), first, we have optimized a method for reprogramming-resistant CD8 T cell clones into T-iPS cells by using sendaiviral vectors. Second, we have optimized stepwise differentiation methods for inducing hematopoietic progenitor cells, T cell progenitors, and functionally matured CD8 single positive CTL. These protocols provide useful in vitro tools and models both for research of antigen-specific T cell immunotherapy and for research of normal and pathological thymopoiesis.

Key words Antigen-specific T cells derived from human iPS cells, In vitro thymopoiesis, Re-differentiation of antigen-specific T cells, T cell receptor rearrangement, T cell immunotherapy

1 Introduction

Peripheral T cells such as CD4 and CD8 single positive αβ T cells play a central role for protecting host from various types of pathogen. CD8 T cells especially exert cytotoxic effector functions to pathogenic cells in HLA-restricted and antigenic peptide-specific manner. The key molecule of T cell side for the recognition is αβ T cell receptor which is generated by recombination of V(D)J gene cluster regions. Although the mechanism permits T cells to have wide antigen specificity, only limited number of antigen-specific T cells exist in patients in chronic disease such as cancer or chronic viral infection. In addition to small pool of antigen-specific T cells, it is well known that continuous TCR stimulation by chronic antigens induces exhaustion of antigen specific T cells by overwork, and it resulted in gradual loss of antigen-specific T cell

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pool [1, 2]. That is one reason why sometime T cell immunity fails to eliminate chronic pathogens and why antigen-specific immunotherapies are desired to develop [3–5]. Very recently, we and another group have reported regeneration of antigen specific T cells via iPS cell ex vivo, as a new proof of concept for restoration of antigen specific T-cell immunity [6, 7]. In this chapter, the methods for in vitro regeneration of antigen-specific T cells from human iPS cells of antigen-specific T cell origin are described.

2 Materials

2.1 Reprogramming T Cells into Pluripotent Stem Cells

1. Antigen-specific T cell clone, T cell line, or tetramer-positive T cells.
2. T cell medium; RPMI1640 medium (Sigma-Aldrich) supplied with 10 % fetal bovine serum (FBS; Invitrogen) and 100 IU/ml of recombinant human interleukin-2 (Peprotech).
3. Anti-CD3/CD28 conjugated magnetic beads (Invitrogen).
4. Phosphate buffer saline Ca^{2+}- and Mg^{2+}- free (PBS; nacalai-tesque, Kyoto, Japan).
5. Retronectin™ (TAKARA, Otsu, Japan).
6. Protamine sulfate (Sigma-Aldrich).
7. Sendai viral vectors containing reprogramming factors (11, or Cytotune™, Dynavec, Tsukuba, Japan).
8. Rotator (TAITEC, Koshigaya, Japan).
9. MCP-1 magnet (Invitrogen).
10. Murine embryonic fibroblast (Japan SLC, Inc., Hamamatsu, Japan).
11. iPS cell medium; DMEM nutrient mixture F-12 HAM (Sigma-Aldrich) supplemented with 20 % knockout serum replacement (KSR; Invitrogen) and 2 mM l-glutamine (Invitrogen), 0.1 mM nonessential amino acid (Invitrogen), 0.1 mM 2-mercaptoethanol (Invitrogen), and 5 ng/ml basic fibroblast growth factor (b-FGF; Wako, Osaka, Japan).
12. Appropriate size of culture dishes, plates, and conical tubes (not specified).

2.2 Differentiation of Hematopoietic Stem/Progenitor Cells from T-iPS Cells [8]

1. T-iPS cells, cultured on irradiated MEF in iPS cell medium.
2. C3H10T1/2 murine stromal cell line (RIKEN Bio-Resource Center, Tsukuba, Japan).
3. Basal Eagle's medium (BME; Invitrogen) supplemented with 10 % FBS (Biological Industries, Kibbutz Beit Haemek, Israel) and 2 mM l-glutamine (Invitrogen).
4. 0.05 % Trypsin-EDTA (Sigma-Aldrich).

5. PBS (nacalai-tesque, Kyoto, Japan).
6. Dissociation solution; PBS supplemented with 0.25 % human trypsin (Invitrogen), 1 mM CaCl (nacalai-tesque, Kyoto, Japan), and 20 % KSR (Invitrogen).
7. Differentiation medium; IMDM (Sigma-Aldrich) with 15 % FBS (AusGeneX, Oxenford, Australia), 100 ng/ml human insulin, 55 ng/ml human transferrin, 50 pg/ml sodium selenite (ITS; Invitrogen), 2 mM l-glutamine (Invitrogen), 50 μg/ml ascorbic acid (nacalai-tesque, Kyoto, Japan), and 0.45 μM α-monothioglycerol (nacalai-tesque, Kyoto, Japan).
8. Human recombinant vascular endothelial growth factor (R&D systems).
9. Human recombinant interleukin-7 (Peprotech).
10. Human recombinant Flt3L (Peprotech).
11. Appropriate size of culture dishes, plates, and conical tubes (not specified).

2.3 Differentiation of Antigen-Specific Cytotoxic T Lymphocytes

1. Hematopoietic progenitor cells induced from T-iPSC.
2. OP9/DL1 murine stromal cell line (RIKEN Bio-Resource Center, Tsukuba, Japan).
3. Peripheral blood mononuclear cells.
4. α-MEM (Invitrogen) supplemented with 20 % FBS (BioWest, Nuaillé, France) and 2 mM l-glutamine (Invitrogen).
5. 0.05 % Trypsin-EDTA (Sigma-Aldrich).
6. PBS (nacalai-tesque, Kyoto, Japan).
7. Human recombinant Flt3L (Peprotech).
8. Human recombinant interleukin-7 (Peprotech).
9. Human recombinant interleukin-15 (Peprotech).
10. Phytohemagglutinin (PHA; Wako, Osaka, Japan).
11. Appropriate size of culture dishes, plates, and conical tubes.

2.4 Characterization of Re-differentiated T Cells by Flowcytometry

1. Re-differentiating or re-differentiated T cells.
2. Peripheral blood mononuclear cells (C.T.L.) for flow cytometry setting.
3. True count tube (BD Bioscience).
4. PBS (nacalai-tesque, Kyoto, Japan) supplemented with 2 % FBS (Invitrogen).
5. Anti-human antibodies CD45-V500 (BD Bioscience), CD5-PE/Cy7 (eBioscience), CD7-PE (Biolegend), CD3-Pacific Blue (eBioscience), CD4-APC/H7 (BD Bioscience), and CD8a-PerCP/Cy5.5 (Biolegend).
6. Propidium iodide (Sigma-Aldrich).

3 Methods

Carry out all culture procedures in appropriate sterile conditions unless otherwise specified.

3.1 Reprogramming of Antigen-Specific T Cells

1. Induce antigen-specific CD8 T cell clones or lines by any methods such as tetramer mediated cell separation and repeated antigen stimulation in vitro.
2. Collect the T cells at 0.5–2.0 × 10^7/ml of RPMI medium supplemented with 10 % FBS in 1.5 ml tube.
3. Prepare appropriate number of CD3/CD28 conjugated beads in another 1.5 ml tube. Wash them twice by PBS, then mixed them with collected T cells in the tube.
4. Rotate the tube for 45–60 min at 10 rpm. Appropriate stimulation allows T cell shape to change into blastic (*see* Fig. 1).
5. Transfer magnetically selected beads and T cell complex into appropriate size of plate and culture them for 48 h in 5 % CO2 incubator. Generally, 0.5–1 × 10^6/ml/cm^2 is recommended for T cell culture.

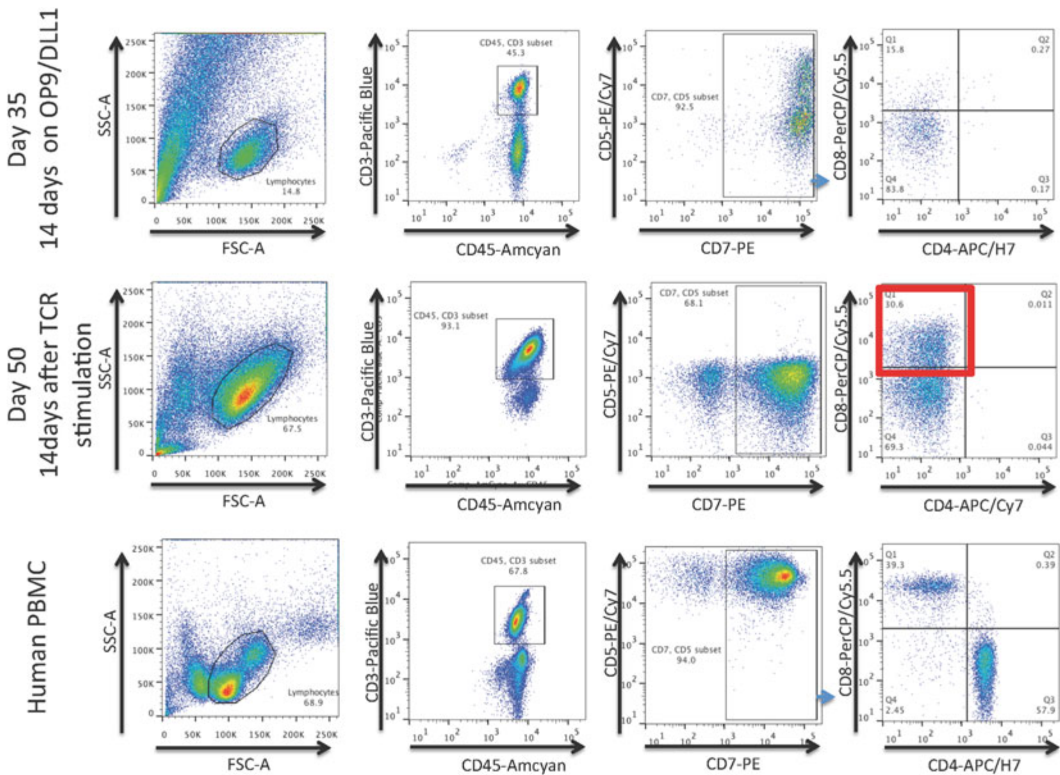

Fig. 1 Fully activated T lymphocytes show blastic shape soon after anti-human CD3/CD28 bead stimulation

6. Resuspend T cells in fresh T cell medium at 1×10^6/ml concentration then transfer them onto retronectin coated well of 24-well plate.
7. Add 10 μg/ml of protamine sulfate and reprogramming factors containing sendai viral vectors at appropriate multiplicity of infection (MOI) to the T cell culture.
8. Centrifuge the 24-well plate at $1000 \times g$ for 30 min at room temperature, and then incubate cells in 5 %CO2 incubator overnight.
9. Wash cells and culture them in fresh complete medium overnight.
10. Wash cells, remove residual beads by magnet, and transfer 1×10^6 T cells onto a 6 cm dish coated with pretreated MEF.
11. Discard half of T cell medium and add fresh pluripotent cell medium once a day till embryonic stem cell like colonies appear around day 21.
12. Isolate single cell-derived colonies and confirm their pluripotency by reported methods [9].

3.2 Induction of Hematopoietic Stem/Progenitor Cells from T-iPSCs [8]

1. Prepare pretreated 10 cm dishes containing confluent 10 T1/2 cell line.
2. Harvest T-iPS cells at confluent from the 6 cm dish. Cells should be recovered as small cell clusters by trypsinization with dissociation solution and pipetting. Transfer 1/10–20 volume of cell cluster onto a pretreated C3H10T1/2 cell layer of 10 cm dish and culture with differentiation medium containing 20 ng/ml VEGF. 30–50 sac-like structures in a 10 cm dish will appear at the final step of the section if they are spread appropriately.
3. Change differentiation medium containing 20 ng/ml VEGF every 2–3 days. The medium from day 7 up to day 14 of culture should contain 20 ng/ml VEGF, 10 ng/ml hFlt-3 L and 1 ng/ml hIL-7.
4. T-iPSC derived sac-like structures containing hematopoietic progenitor cells appear around day 10 of culture.

3.3 Induction of Antigen-Specific CD8 T Cells

The following method should be started around day 14 of the culture to have better recovery of hematopoietic progenitor cells.

1. Prepare pretreated 6 cm dishes containing confluent OP9/DL1 cell line.
2. Take out hematopoietic progenitor cell suspension by breaking the sac-like structures with mechanical stress, concretely, scratching them by cell scraper and pipetting.
3. Transfer hematopoietic cell containing medium into a new 50 ml tube through 40 μm cell strainer and centrifuge the cells at $400 \times g$ for 10 min.

4. Resuspend hematopoietic cells in fresh T cell differentiation medium supplemented with 10 ng/ml hFlt-3 L and 1 ng/ml hIL-7, and transfer them onto pre-irradiated 6 cm dishes of OP9/DL1 stromal cell line.
5. Change half of medium every 2–3 days. The culture medium is supplied with 10 ng/ml hFlt-3 L and 1 ng/ml hIL-7 up to day 21 of culture on OP9/DL1.
6. CD7 positive TCR-CD3 complex positive T-lineage cells appear until day 21 of culture. Expression level of CD5, CD4, and CD8 may vary among T-iPSC clone at this time point (*see* **Notes**).
7. Collect CD45 and TCR-CD3 complex positive cells from floating cells in culture by flow cytometry or magnetic bead selection.
8. Stimulate the TCR-CD3 complex positive T-lineage cells by 1–5 μg/ml PHA in the presence of 10 ng/ml IL-7, 10 ng/ml IL-15, and irradiated PBMCs. 5×10^6 irradiated PBMC in a well of 48-well plate is used to stimulate sorted cells up to 1×10^6. Size of cell culture could be modified according to number of sorted CD3 T-lineage cells.
9. CD8 single positive T cells appear until day 14 after stimulation. After this stage, antigen specificity and function of regenerated CD8 T cells can be evaluated by common methods for testing CTL clones (tetramer binding assay, ELISPOT, 51Cr release assay, and so on).

3.4 Characterization of Antigen-Specific CD8 T Lymphopoiesis by Flow Cytometry

1. Harvest 2×10^5 differentiating T cells in culture at desired time point.
2. Wash and suspend harvested cells in 200 μL of PBS supplemented with 2 % FBS.
3. Add indicated anti-human monoclonal antibody-cocktail to the cell suspension, and then incubate for 25 min on ice in the dark.
4. Wash cell twice by 1 ml of cold PBS supplemented with 2 % FBS.
5. Suspend labeled cells in PBS supplemented with 2 % FBS and 1 μg/ml propidium iodide.
6. Now labeled-T cells are ready for flow cytometoric analyses and cell sorting.

4 Notes

1. Freshly isolated polyclonal T cells can be reprogrammed as ease by both retroviral vectors or sendaiviral vectors containing Yamanaka factors [6]. SV40 large T antigen would be necessary for reprograming antigen-specific T cell clones that have been stimulated repeatedly in vitro by specific antigens.

2. Both low and high MOI of sendaiviral vectors result in low recovery of T-iPSC colonies by low transduction efficiency and low viability of cells, respectively [10].
3. Confluent MEF, C3H10T1/2, and OP9/DL1 layered dishes should be treated by 50 Gy irradiation or mitomycin C to inhibit further proliferation of those feeder cells in co-culturing steps.
4. Transferring various number of transduced T cells onto different MEF dishes would be helpful because induction efficiency of T-iPS cell may vary by status of T cell clones, transduction efficiencies, and many factors.
5. In our observation, T-iPS cells differentiate more effective into T-lineage cells than the other types of pluripotent stem cells without pre-rearrangement of TCR genes when they are cultured by the protocols.
6. In our observation, extensive culture of re-differentiating T cells on OP9/DL1 increase a chance of additional rearrangement of TCRA gene at CD4 and CD8 double-positive stage, which results in loss of antigen specificity of the TCR. Preliminary experiments using individual T-iPS cell clones are recommended to optimize the timing of TCR stimulation to induce functionally mature CD8 single positive T cells inhibiting RAG recombinase reactivation-mediated additional rearrangement of TCRA gene.

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