Published in final edited form as:

Methods Mol Biol. 2019 ; 2048: 131–141. doi:10.1007/978-1-4939-9728-2_14.

In Vitro Differentiation of T Cells from Murine Pluripotent Stem Cells

Xiaoniao Chen^{#1,2}, Fengyang Lei^{#2}, Liqiang Wang¹, Xiaofang Xiong³, Jianxun Song^{3,*}

¹Department of Ophthalmology, Chinese PLA General Hospital, Beijing, China

²Department of Ophthalmology, Harvard Medical School, Boston, MA, USA

³Department of Microbial Pathogenesis and Immunology, Texas A&M University, Bryan, TX, USA

These authors contributed equally to this work.

Abstract

In recent years cancer immunotherapy, especially the cell-based immunotherapy, has reached several milestones and achieved a lot of cancer remission in the clinics. Obtaining a more potent and effective cytotoxic T lymphocytes (CTLs) for cancer immunotherapy is always the ultimate goal for the researchers. However, the difficulty in harvesting a large number of tumor antigen-specific CTLs from the tumor patient is still a major obstacle we need to overcome. In our previous studies, it is shown that pluripotent stem cell-derived CTL—especially the genetically engineered antigen-specific CTLs—may serve as a good source of unlimited number of highly reactive and antigen-specific CTLs. Here we present a two-step method for the generation of antigen-specific T lymphocytes from iPS cells by in vitro priming and in vivo maturation.

Keywords

Stem cell biology; T cells; Induced pluripotent stem cells; Differentiation; Notch signaling

1 Introduction

T cell-based cancer immunotherapy has achieved a lot of promising results in recent years [1]. Cytotoxic T lymphocytes (CTLs), especially the tumor antigen-specific CTLs, can recognize malignant tumor cells by precisely interacting tumor antigens with the T cell receptors (TCR) and release cytotoxins (perforin and granzyme B) as well as cytokines (IFN- γ , TNF- α) to kill malignant cells. It is known that a less-differentiated and central-memory-like CTL is the desiring arsenal for T cell-based immunotherapy, because less-differentiated CTLs have a higher potential for proliferation, are less susceptible to apoptosis than terminally differentiated CTL, and have a more sensitive response to homeostatic cytokines [2–6]. However, it is impossible to obtain this rare population of CTLs from patients, especially the tumor patients whose immune system is significantly suppressed. We have taken a different approach to generate this highly reactive and antigen (Ag)-specific CTLs for the T cell cancer immunotherapy.

* jus35@tamu.edu.

Overexpressing a unique TCR to the self-renewable stem cells for immune reconstitution builds up a therapeutic potential for treating a variety of diseases [7–9]. However, there is another impossibility to obtain embryonic tissue from tumor patients. Although hematopoietic stem cells (HSCs) are good candidates for therapeutic purposes, which have been widely applied in clinic [10–12], HSCs have reduced differentiation and proliferative capacities following ex vivo isolation, and HSCs are difficult to expand in vitro [13, 14]. Recently the introduction of iPS cell technology has given us a third path to obtain pluripotent cells that could be applied for many therapeutic purposes. Furthermore, iPS bears an indefinite capability of in vitro proliferation and directed differentiation under certain conditions. Therefore, we believe that iPS cells have a greater potential to be used in the cellular immunotherapy for cancer.

Here, we present a two-step method for the generation of antigen-specific T lymphocytes from iPS cells by in vitro priming and in vivo maturation. In vitro stimulation with a Notch ligand Delta-1 drives early stage T cell differentiation from iPS cells, and TCR gene transduction results in iPS cells maturing into antigen-specific T cells in vivo, which shows a strong antigen reactivity. In general, we demonstrate antigen-specific CTL is able to differentiate from iPS cell. Our studies provide a potentially efficient and feasible approach for generating antigen-specific CTLs for T cell-based cancer immunotherapies and facilitate the development of therapeutic strategies for diseases [15–17].

2 Materials

2.1 Mice/Cell Lines

1. iPS-MEF-Ng-20D-17 cell line is kind gift from Dr. Shinya Yamanaka at Kyoto University [18]. It was generated from mouse embryonic fibroblasts by retroviral transduction of a combination of *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* genes. iPS cells are routinely cocultured with irradiated SNL76/7 feeder cells supplemented with 15% FBS-DMEM medium.
2. OP9 and OP9-DL1 cell lines are generous gifts from Dr. Juan Carlos Zuniga-Pflucker at the University of Toronto [19]. OP9 and OP9-DL1 cell lines are routinely maintained in 20% FBS- α -MEM medium.
3. SNL76/7 cell line (ATCC) is maintained in 10% FBS-DMEM medium. Prior to coculture with iPS cells, SNL76/7 cells will undergo a 5000 Rads irradiation in the ^{60}Co irradiator, and the irradiated cells are designated irSNL76/7.
4. Mice: 6–8 weeks of age female C57/BL6J and B6.127S7-*Rag1*^{tm1Mom}/J mice are purchased from the Jackson Laboratory.
5. Platinum-E (Plat-E) retroviral packaging cells (*see* Note 4).

2.2 Cell Culture

1. 6- and 24-well culture plate (BD).

⁴Wash the OP9-DL1 feeding layer with 10 mL PBS one more time to get the maximal recovery of partially differentiated iPS cells.

2. 100 mm culture dish (BD).
3. 70 μ m cell strainer (BD).
4. 1 mL and 10 mL syringes (BD).
5. 27 G^{1/2} and 18 G^{1/2} needles (BD).
6. Various plastic pipettes (5 mL, 10 mL, 25 mL, 50 mL; BD).
7. 0.22 μ m bottle top filter (Corning).
8. 0.4 μ m filter (Millipore).
9. Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen).
10. α -Minimal Essential Medium (α -MEM) (Invitrogen): with one pack of α -MEM powder, 2.2 g NaHCO₃ and 1 L of ddH₂O are added. Filtration of prepared medium through a 0.22 μ m bottle top filter is mandatory for sterilization. FBS, antibiotics, and cytokines are added into the medium accordingly.
11. Phosphate-buffered saline (PBS): 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na₂HPO₄·2H₂O, 2 mmol/L KH₂PO₄, pH adjusted to 7.4, and autoclaved before use.
12. 100 $\times$ penicillin and streptomycin mix (10,000 U/mL, Invitrogen).
13. Fetal bovine serum (FBS, heat-inactivated, HyClone).
14. Flt-3 ligand (Flt-3 L, PeproTech).
15. Interleukin 7 (IL-7, PeproTech).
16. Gelatin (Sigma-Aldrich).
17. Brefeldin A (Invitrogen).
18. Fc blocker 24G2 (BD).
19. Permeabilizing kit (BioLegend).

2.3 Retroviral Transduction

1. GeneJammer transfection reagent (Stratagene).
2. 5 μ g/mL polybrene (Sigma-Aldrich).
3. FACS Aria SORP cell sorter (BD).

2.4 Peptide Stimulation

1. CD8⁺ MACS separation system (Miltenyi Biotec).
2. OVA_{257–264} peptide (GenScript).

2.5 PCR Assay

1. DNeasy Blood and Tissue DNA isolation kit (Qiagen).
2. PCR Master Mix Kit (Qiagen).

3. *OT-I* forward primer: 5'-ACGTGTATTCCCATCTCTGGA CAT-3'.
4. *OT-I* reserve primer: 5'-TGTTTCATAATTGGCCCGAGAG CTG-3'.

2.6 Antibodies

1. CD4: GK1.5 (BioLegend).
2. CD8: 6A242 (Santa Cruz).
3. TCR V β 5: MR9-4 (BioLegend).
4. TCR V β : H57-597 (BioLegend).
5. CD3: 17A2 (BioLegend).
6. CD45: 30-F11 (BioLegend).
7. CD117: 2B8 (BioLegend).
8. IL-2: JES6-5H4 (BD Pharmingen).
9. IFN- γ : XMG1.2 (BD Pharmingen).

3 Methods

3.1 Generation of Retroviral Vector

1. MSCV-IRES-DsRED (MiDR) vector is designed and constructed based on MSCV-IRES-GFP vector by replacing the GFP module with a DsRED reporter module.
2. OT-I T cell receptor V α 2 chain and V β 5 chain are individually subcloned and linked by a 2A sequence before inserting into the MiDR vector to make OT-I/MiDR construct.

3.2 Retroviral Transduction and Cell Sorting

1. Plat-E packaging cells are used to generate pseudovirus that will be used for the transduction. On Day -3, 3×10^6 Plat-E cells are seeded on a 100 mm petri dish one day prior to transfection.
2. On Day -2, Plat-E cells will be transfected with OT-I MiDR vector by using GeneJammer transfection reagent.
3. On Day -1, 1×10^6 iPS cells will be seeded into one well of a 1% gelatin precoated 24-well plate (see Note 1).
4. Transduction will be performed by spinning at $1000 \times g$ for 1 h at 32 °C in the presence of 5 μ g/mL polybrene on Day 0.
5. After transduction, plates will be placed in 32 °C, 5% CO₂ incubator overnight to further promote the retroviral integration.

¹Precoat the plate with 0.1% gelatin at 4 °C overnight.

6. On Day 1, repeat the transduction as described above. A 6-well plate will be precoated with irSNL76/7 feeder cells for future use.
7. On day 2, transduced iPS cells will be harvested by trypsin, centrifuged at 800 rpm for 5 min, and seeded on the precoated irSNL76/7 feeder cells.
8. At confluency, cells will be harvested, centrifuged at 800 rpm for 5 min, and processed for cell sorting. GFP and DsRED double positive cells will be sorted by FACS Aria cell sorter. Sorted cells will be continuously cultured on irSNL76/7 feeder cells for future use.

3.3 In Vitro Priming with OP9-DL1 Cells

1. On Day 0, 5×10^4 iPS cells will be seeded on a 100 mm culture dish containing confluent OP9-DL1 cell monolayer in 20% FBS α -MEM media (*see Note 2*).
2. On Day 3, change culture medium.
3. On Day 5, cells will be harvested and centrifuged at 800 rpm for 5 min before incubating on a fresh 100 mm culture dish for 30 min in 37 °C incubator (*see Note 3*). Floating cells will be collected and counted; 5×10^5 cells will be transferred to a fresh culture dish containing confluent OP9-DL1 cell monolayer in 20% FBS α -MEM media. Cytokine mFlt-3 L (final concentration, 5 ng/mL) will be added in the culture.
4. On Day 8, loosely attached cells will be gently pipette off (*see Note 4*).
5. After harvesting cells from the coculture, cells will be centrifuged at 800 rpm for 5 min and resuspended in 20% FBS α -MEM media supplemented with mFlt-3 L (5 ng/mL) and mIL-7 (1 ng/mL).
6. Cells will be transferred into a 6-well culture plate coated with confluent OP9-DL1 cells (*see Note 5*).
7. From day 10, culture media will be changed in every other day (20% FBS α -MEM media supplemented with mFlt-3 L (5 ng/mL) and mIL-7 (1 ng/mL)).
8. Culture plates coated with feeder OP9-DL1 cells will be changed in 4–6 days depending on the growth of the feeder cells.

3.4 In Vitro Analysis

1. Morphological changes of differentiated cells and cell recovery rates (Fig. 1).
2. Gene integration and expression are analyzed by both PCR and RT-PCR analyses.
3. At different days of coculture with OP9-DL1 cells, live cell images are taken by phase-contrast microscope.

²To successfully induce the T cell differentiation, a confluent monolayer of OP9-DL1 cells is required.

³Sitting at 37 °C for 30 min is to remove the excessive OP9-DL1 cells.

⁵Usually iPS cells recovered from one 100 mm culture dish will be transferred into one well of the 6-well plate.

4. Cell recovery rates are calculated based on the number of cells that harvested from culture (Fig. 2a).
5. At different days of coculture, cells will be removed from culture by trypsinization and washed with cold PBS before proceeding to cell surface staining.
6. Before staining with different fluorochrome-conjugated antibodies, cells are blocked by Fc blocker 24G2 in 4 °C for 20 min.
7. After staining in 4 °C for 20 min, cells will be washed three times in cold PBS before loading on flow cytometer.
8. Flow cytometric analysis of surface marker changes (Fig. 3a).
9. Activation of in vitro differentiated iPS cells (Fig. 3b).
10. One day before activation assay, precoat a 24-well plate with anti-CD3 (final concentration, 4 µg/mL, in PBS) at 4 °C overnight.
11. On day 22 of coculture with OP9-DL1 cells, iPS cell-derived T cells are harvested from culture and washed with cold PBS before stimulating with plate-coated anti-CD3 and soluble anti-CD28 antibodies (final concentration, 4 µg/mL).
12. Incubating plates in 37 °C, 5% CO₂ incubator for 40 h and then Brefeldin A is added into culture for another 4 h (*see* Note 6).
13. At the end of stimulation, cells are harvested, washed, and blocked by Fc blocker as described above. Prepared cells are stained for surface markers as CD8 and TCR Vβ chain by using fluorochrome-conjugated antibodies.
14. After surface staining, cells are fixed by using 4% formaldehyde and permeabilized by using BioLegend's Permeabilizing kit.
15. After permeabilization, intracellular molecules IL-2 and IFN-γ are stained by using different fluorochrome-conjugated antibodies.
16. Before final flow cytometric examination, cells are washed three times in cold PBS to exclude excessive antibodies.

3.5 In Vivo Maturation

1. On Day 22 of coculture, partially differentiated iPS cells are trypsinized off, centrifuged at 800 rpm for 5 min, and incubated on a fresh culture dish in 37 °C for 30 min (*see* Note 7).
2. Floating cells will be collected, passed through a 70 µm nylon strainer to exclude cell clumps, and washed three times with cold PBS (*see* Note 8).

⁶Brefeldin A is commonly used in intracellular staining to retain the synthesized cytokine molecules.

⁷37 °C, 30 min incubation is to exclude OP9-DL1 cells.

⁸Large cell clump will cause pulmonary embolism to mice.

3. Cells will be resuspended maintained in cold PBS with a concentration of 1.5×10^7 cells/mL before injection.
4. 200 μ L cell suspension or 3×10^6 cells are adoptively transferred into 4-week-old B6.129S7-*Rag1^{tm1Mom}/J* mice through the tail vein. Three weeks are allowed for in vivo maturation of partially differentiated iPS cells (see Note 9).
5. Maturation in *Rag1^{-/-}* mice. After three weeks of in vivo development, *Rag1^{-/-}* mice are sacrificed; spleen and lymph nodes are removed from mice (Fig. 4a).
6. Single cells are processed through mechanical breakdown. Red blood cells are lysed by ACK lysis buffer and mononucleocytes are collected and washed twice in cold PBS.
7. After washing, cells are blocked with Fc blocker 24G2 in 4 °C for 20 min and before staining with different fluorochrome-conjugated anti-CD3, anti-CD4, anti-CD8, and anti-TCR β antibodies in 4 °C for 20 min.
8. At the end of staining, cells are washed three times in cold PBS before flow cytometric analysis.

3.6 Ex Vivo Analysis

1. Three weeks after in vivo development, a large population of CD8+TCRV β 5+ cells were present in the lymphoid organs of *Rag1^{-/-}* mice received in vitro primed OT I-iPSCs as compared with other controls.
2. Sacrifice the mice and collect lymphoid organs such as spleen and lymph nodes from the mice.
3. Harvest CD8+ T cells by negative selection and stimulate the CD8+ T cells with OVA_{257–264} peptide.
4. After stimulation, intracellular staining of IL-2 and IFN- γ is performed as described above.
5. At the end of the staining, wash cells in cold PBS twice and analyze the cytokine profile by flow cytometry (Fig. 4b).

Acknowledgments

This project was funded, in part, under the grants from National Institute of Health Grant R01AI121180, R21AI109239, and R01CA221867 and the American Diabetes Association (1-16-IBS-281) to J.S.

References

1. Schuster SJ, Svoboda J, Chong EA, Nasta SD, Mato AR, Anak O, Brogdon JL, Pruteanu-Malinici I, Bhoj V, Landsburg D, Wasik M, Levine BL, Lacey SF, Melenhorst JJ, Porter DL, June CH (2017) Chimeric antigen receptor T cells in refractory B-cell lymphomas. *N Engl J Med* 377(26):2545–2554. 10.1056/NEJMoa1708566 [PubMed: 29226764]

⁹Before IV injection, mice will be placed under an infrared light to dilate their tail vein which will facilitate the injection.

2. Hataye J, Moon JJ, Khoruts A, Reilly C, Jenkins MK (2006) Naive and memory CD4+ T cell survival controlled by clonal abundance. *Science* 312(5770):114–116. 10.1126/science.1124228 [PubMed: 16513943]
3. Seki Y, Yang J, Okamoto M, Tanaka S, Goitsuka R, Farrar MA, Kubo M (2007) IL-7/STAT5 cytokine signaling pathway is essential but insufficient for maintenance of naive CD4 T cell survival in peripheral lymphoid organs. *J Immunol* 178(1):262–270 [PubMed: 17182563]
4. Stemmerger C, Huster KM, Koffler M, Anderl F, Schiemann M, Wagner H, Busch DH (2007) A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. *Immunity* 27(6):985–997. 10.1016/j.immuni.2007.10.012 [PubMed: 18082432]
5. Siewert C, Lauer U, Cording S, Bopp T, Schmitt E, Hamann A, Huehn J (2008) Experience-driven development: effector/memory-like alphaE+Foxp3+ regulatory T cells originate from both naive T cells and naturally occurring naive-like regulatory T cells. *J Immunol* 180(1):146–155 [PubMed: 18097014]
6. Hinrichs CS, Borman ZA, Gattinoni L, Yu Z, Burns WR, Huang J, Klebanoff CA, Johnson LA, Kerkar SP, Yang S, Muranski P, Palmer DC, Scott CD, Morgan RA, Robbins PF, Rosenberg SA, Restifo NP (2011) Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. *Blood* 117 (3):808–814.10.1182/blood-2010-05-286286 [PubMed: 20971955]
7. Alajez NM, Schmielau J, Alter MD, Cascio M, Finn OJ (2005) Therapeutic potential of a tumor-specific, MHC-unrestricted T-cell receptor expressed on effector cells of the innate and the adaptive immune system through bone marrow transduction and immune reconstitution. *Blood* 105 (12):4583–4589. 10.1182/blood-2004-10-3848 [PubMed: 15746083]
8. Yang L, Baltimore D (2005) Long-term in vivo provision of antigen-specific T cell immunity by programming hematopoietic stem cells. *Proc Natl Acad Sci U S A* 102(12):4518–4523. 10.1073/pnas.0500600102 [PubMed: 15758071]
9. Zhao Y, Parkhurst MR, Zheng Z, Cohen CJ, Riley JP, Gattinoni L, Restifo NP, Rosenberg SA, Morgan RA (2007) Extrathymic generation of tumor-specific T cells from genetically engineered human hematopoietic stem cells via notch signaling. *Cancer Res* 67 (6):2425–2429.10.1158/0008-5472.CAN-06-3977 [PubMed: 17363559]
10. Boztug K, Schmidt M, Schwarzer A, Banerjee PP, Diez IA, Dewey RA, Bohm M, Nowrouzi A, Ball CR, Glimm H, Naundorf S, Kuhlcke K, Blasczyk R, Kondratenko I, Marodi L, Orange JS, von Kalle C, Klein C (2010) Stem-cell gene therapy for the Wiskott-Aldrich syndrome. *N Engl J Med* 363(20):1918–1927. 10.1056/NEJMoa1003548 [PubMed: 21067383]
11. Mendez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, Scadden DT, Ma'ayan A, Enikolopov GN, Frenette PS (2010) Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466(7308):829–834. 10.1038/nature09262 [PubMed: 20703299]
12. Peerani R, Zandstra PW (2010) Enabling stem cell therapies through synthetic stem cell-niche engineering. *J Clin Invest* 120(1):60–70. 10.1172/JCI41158 [PubMed: 20051637]
13. Boitano AE, Wang J, Romeo R, Bouchez LC, Parker AE, Sutton SE, Walker JR, Flaveny CA, Perdew GH, Denison MS, Schultz PG, Cooke MP (2010) Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science* 329 (5997):1345–1348. 10.1126/science.1191536 [PubMed: 20688981]
14. Himburg HA, Muramoto GG, Daher P, Meadows SK, Russell JL, Doan P, Chi JT, Salter AB, Lento WE, Reya T, Chao NJ, Chute JP (2010) Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. *Nat Med* 16(4):475–482. 10.1038/nm.2119 [PubMed: 20305662]
15. Lei F, Zhao B, Haque R, Xiong X, Budgeon L, Christensen ND, Wu Y, Song J (2011) In vivo programming of tumor antigen-specific T lymphocytes from pluripotent stem cells to promote cancer immunosurveillance. *Cancer Res* 71(14):4742–4747.10.1158/0008-5472.CAN-11-0359 [PubMed: 21628492]
16. Lei F, Haque M, Sandhu P, Ravi S, Song J, Ni B, Zheng S, Fang D, Jia H, Yang JM, Song J (2017) Development and characterization of naive single-type tumor antigen-specific CD8+ T lymphocytes from murine pluripotent stem cells. *Oncoimmunology* 6(7):e1334027 10.1080/2162402X.2017.1334027 [PubMed: 28811978]

17. Lei F, Haque R, Weiler L, Vrana KE, Song J (2009) T lineage differentiation from induced pluripotent stem cells. *Cell Immunol* 260 (1):1–5. 10.1016/j.cellimm.2009.09.005 [PubMed: 19811778]
18. Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. *Nature* 448(7151):313–317. 10.1038/nature05934 [PubMed: 17554338]
19. Schmitt TM, Zuniga-Pflucker JC (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* 17(6):749–756 [PubMed: 12479821]

Fig. 1. Morphology of iPS cell under differentiation. (a) At various days, mouse iPS cells are imaged (scale bars, 50 μ m). (b) RT-PCR and (c) PCR characterization of OT-I TCRV β 5 gene integration and expression

Fig. 2. iPS cell differentiation in the presence of Notch signaling. **(a)** Cell recovery of iPS cell-derived cells. Numbers of iPS cell-derived cells present on day 0 are assigned a value of 100%, and numbers of cells on days 7, 14, and 21 are used to calculate the percentage recovery relative to day 0. Data represent the mean $\pm$ SEM. **(b)** Changes in hematopoietic marker CD117 and CD45 expression in iPS cells (GFP+). Expression of each marker was determined at the indicated days. Data represent the mean $\pm$ SEM

Fig. 3.

T cell differentiation from iPS cells. Mouse iPS cells are cocultured with OP9-DL1 cells as described in Fig. 1. On day 22, iPS cell-derived cells were isolated and analyzed. **(a)** CD4⁺ CD8⁻ or CD4⁻ CD8⁺ cells after gating on CD3⁺ and TCR β ⁺ populations. **(b)** Cells are stimulated with plate-coated anti-CD3 and soluble anti-CD28 antibodies for 40 h at 37 °C at 5% CO₂. IL-2 and INF- γ are analyzed by intracellular staining, after gating on live CD4⁺ CD8⁻ T cells (dark lines; shaded areas indicate isotype controls)

Fig. 4.

Antigen-specific CD8⁺ T cell development from iPS cells matured in Rag1^{-/-} mice. In vitro primed OT-I TCR gene-transduced iPS cells are injected IV into Rag1^{-/-} mice. After 3 weeks, OVA-specific CD8⁺Vβ5⁺ T cell development is determined. (a) CD8⁺Vβ5⁺ T cells from pooled lymph nodes and spleen are analyzed by flow cytometry, after gating on CD8⁺ populations. (b) IL-2 and INF-γ production (dark lines; shaded areas indicate isotype

controls) are determined by intracellular cytokine staining, after gating on the CD8+V β 5+ populations

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript