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Generation of Tumor Antigen-Specific Cytotoxic T Lymphocytes from Pluripotent Stem Cells

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Abstract

Immunotherapy is a developing but very promising arsenal to treat cancer. Acquiring a more potent and effective approach in cancer immunotherapy is always the ultimate pursuance. CTL-based therapies are highly acclaimed recently due to its direct killing property. However, difficulty in obtaining adequate number of CTLs is still a major obstacle. In previous studies, it is shown that pluripotent stem cell-derived cytotoxic T lymphocytes (CTL)—especially the genetically engineered tumor antigen-specific CTLs—may serve as a good candidate for this goal. Here we introduce a novel approach in generating tumor antigen-specific CTLs from induced pluripotent stem cells (iPSCs) by using both in vitro and in vivo priming mechanisms for the tumor management in a murine melanoma model.

Keywords

Cytotoxic T lymphocytes; Notch signaling; Immunotherapy; Melanoma; Pluripotent stem cells

1 Introduction

In recent years, from an in-depth understanding of the immune system, cancer immunotherapy has been developed to treat patients with a high efficacy. Within the concept of immunotherapy, the adoptive cell transfer (ACT)-based therapy has achieved a significant tumor regression in patients with metastatic tumors [1].

The development and clinical application of chimeric antigen receptor T (CAR-T) cell is an exceptional success that falls into the category of ACT therapy [2].

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However, there are major issues of using mature, autologous T cells that are isolated from cancer patient, including T cell anergy and/or exhaustion [3]. An alternative approach to obtain unlimited amount of functional T cells for ACT therapy is to generate induced T cells from stem cells. Several groups, including our own, have reported that antigen-specific T lymphocytes could be generated from stem cells for the following immunotherapy [4, 5]. Concerns about nonspecific differentiation and extended in vivo development of stem cell-derived, antigen-specific T cells are still existing. To minimize these impacts, we have developed an in vitro coculture system to prime the T cell receptor (TCR) harboring stem cell toward T lineage differentiation before adoptive transfer. In this study, we first overexpressed the induced pluripotent stem cells (iPSCs) with a TCR recognizing the tyrosinase-related protein 2 (TRP2; a protein extensively expressed by melanocytes and melanoma) by retroviral-mediated gene delivery. Subsequently, TRP2-iPSCs were primed on OP9 cells overexpressing Notch ligands Delta-like 1 and 4 (DL1 and 4), which are critical signals for T lymphocyte development. We demonstrated that TRP2-specific CD8⁺ T cells could be induced from the TRP2-iPSCs by coculturing with OP9-DL1/DL4 cells. Furthermore, OP9-DL1/DL4-primed TRP2-iPSCs are able to fully develop and differentiate into TRP2-specific CD8⁺ cells in the recipient mice and suppress the growth of engrafted melanoma.

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. It was generated from mouse embryonic fibroblasts by retroviral transduction of a combination of *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc* genes [6] (see Note 1). iPS cells are routinely cocultured with irradiated SNL76/7 feeder cells supplemented with 15% FBS-DMEM medium.
2. OP9, OP9-DL1, OP9-DL4, and OP9-DL1/DL4 cell lines (see Note 2): OP9 and OP9-DL1 cell lines are generous gifts from Dr. Juan Carlos Zuniga-Pflucker at University of Toronto [7]. OP9-DL4 cells and OP9-DL1/DL4 cells are generated by retroviral introduction of either *DL4* gene or *DL1/DL4* fusion gene (see Note 3) into OP9 cells [8]. OP9, OP9-DL1, OP9-DL4, and OP9-DL1/DL4 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 ⁶⁰Co irradiator, and the irradiated cells are designated irSNL76/7 [4].
4. Mice: 6–8 weeks of age female C57/BL6J mice are purchased from Jackson Laboratory.

¹iPS-MEF-Ng-20D-17 cell line is a kind gift from Dr. Shinya Yamanaka at the Kyoto University through the RIKEN cell bank via a completed material transfer agreement (MTA) [6].

²OP9-DL1 cell line is a kind gift from Dr. J.C. Zuniga-Pflucker at the University of Toronto via a completed material transfer agreement (MTA) [7].

³DL1 and DL4 constructs are obtained from Dr. Freddy Radtke at the Swiss Institute for Experimental Cancer Research via a completed material transfer agreement (MTA) [8].

5. Platinum-E (Plat-E) retroviral packaging cells [9] (*see* Note 4).
6. B16-F10 cells (ATCC): are maintained in 10% FBS-DMEM medium.

2.2 Cell Culture

1. 6- and 24-well culture plate (BD).
2. 100 mm culture dish (BD).
3. 70 μ m cell strainer (BD).
4. 1 and 10 mL Syringes (BD).
5. 27G^{1/2} and 18G1^{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× Penicillin and Streptomycin Mix (10,000 U/mL, Invitrogen).
13. Fetal bovine serum (FBS, heat-inactivated, HyClone).
14. Flt-3 ligand (Flt-3L, PeproTech).
15. Interleukin 7 (IL-7, PeproTech).
16. Neutral Formalin buffer (Decal Chemical).
17. Gelatin (Sigma-Aldrich).
18. Brefeldin A (Invitrogen).
19. Formaldehyde solution (Sigma-Aldrich).
20. Fc blocker 24G2 (BD).
21. Permeabilizing kit (BioLegend).

2.3 Retroviral Transduction

1. GeneJamma transfection reagent (Stratagene).

⁴. Plat-E cells are constructed based on the 293T cell line to permanently express retroviral packaging signals *env* and *gag-pol* proteins [9].

2. 5 µg/mL polybrene (Sigma-Aldrich).
3. FACS Aria SORP cell sorter (BD).

2.4 Murine Melanoma Model

1. 1 mL Syringes (BD).
2. PBS (pH 7.4).

2.5 Peptide Stimulation

1. CD8⁺ MACS separation system (Miltenyi Biotec).
2. TRP2_{180–188} peptide (GenScript).

2.6 PCR Assay

1. DNeasy Blood and Tissue DNA isolation Kit (Qiagen).
2. PCR Mastermix Kit (Qiagen).
3. *TRP2* forward primer: 5'-ATTTTAGATCTCCACCATG CTGAT TCTAAGCCTGTTG-3'.
4. *TRP2* reverse primer: 5'-TAAGAATTCTCAGGAATTTT TTTTCTTGAC-3'.

2.7 Immunoblotting

1. RIPA lysis buffer (G-Biosciences).
2. Bio-Rad DC Protein Assay (Bio-Rad).
3. Western Blot NuPAGE system (Invitrogen).
4. ECL immunodetection system (Amersham Pharmacia Biotech).

2.8 Histology and Immunofluorescence

1. Formalin solution (ThermoFisher).
2. 3% Hydrogen peroxide.
3. DAPI reagent (Enzo Life Sciences).
4. Hematoxylin and eosin (HE) (Sigma-Aldrich).
5. Xylene (Sigma-Aldrich).
6. Ethanol.
7. Blocking buffer: 3% bovine serum albumin (BSA) in PBS.

2.9 Antibodies

Antibodies are listed in Table 1.

3 Methods

3.1 Generation of Retroviral Vector and Transduction (Fig. 1)

1. Construct MSCV-IRES-DsRED (MiDR) vector from MSCV-IRES-GFP vector by replacing the GFP gene with the DsRED gene (*see Note 5*).
2. Insert TRP2-specific TCR gene ($V\alpha 17$ and $V\beta 11$ linked by a P2A sequence) into the MiDR vector to make a TRP2-MiDR construct.

3.2 Retroviral Transduction and Cell Sorting

1. Platinum-E (Plat-E) packaging cell line is used to generate a pseudovirus for following retroviral transduction.
2. Seed 3×10^6 Plat-E cells into a 100 mm culture dish 1 day prior to plasmid transfection.
3. On day 0, transfect Plat-E cells with TRP2-MiDR retroviral vector by using GeneJamma transfection reagent.
4. On day 1, seed 1×10^6 iPS cells into one well of a 0.1% gelatin-coated 24-well plate.
5. On day 2, collect pseudovirus-containing supernatant from transfected Plat-E culture and pass the supernatant through a $0.4 \mu\text{m}$ filter to exclude potential debris and contaminants.
6. Perform retroviral transduction in a centrifuge at $500 \times g$ for 1 h in the presence of $5 \mu\text{g/mL}$ polybrene at 32°C .
7. After transduction, place cells in a 32°C , 5% CO_2 incubator overnight.
8. On day 3, repeat the second transduction as described above. Meanwhile, pre-coat a 6-well plate with irSNL76/7 feeder cells for future use.
9. On day 4, collect transduced iPS cells by trypsinization, centrifuge at $400 \times g$ for 5 min, and seed into the 6-well plate pre-coated with irSNL76/7 feeder cells.
10. At confluence, collect cells by trypsinization, centrifuge at $400 \times g$ for 5 min, and process for cell sorting. GFP and DsRED double positive cells will be sorted by a FACS Aria SORP cell sorter. Culture sorted cells (TRP2-iPSC) on fresh irSNL76/7 feeder cells for future use [10] (*see Note 6*).
11. OP9-DL4 and OP9-DL1/DL4 cell lines are generated as described above but by using DL4-MiG and DL1/DL4-MiG constructs, respectively. Transduced cells are sorted by FACS Aria SORP based on GFP expression.

⁵The detailed information about retroviral vector and gene manipulation belongs to the area of molecular cloning. It is beyond the scope of this study and will not be further discussed.

⁶For cell sorting, it is mandatory to pass the cells through a cell strainer several times to prevent machine clogging.

3.3 Coculture with OP9, OP9-DL1, OP9-DL4, and OP9-DL1/DL4 Cell Lines

1. On day 0, seed 5×10^4 TRP2-iPSCs into a 100 mm culture dish coated with a confluent monolayer of OP9 (or other OP9-related cell lines) cells in 20% FBS α -MEM medium.
2. On day 3, change culture medium.
3. On day 5, collect cells by trypsinization and centrifuge at $400 \times g$ for 5 min before incubating on a fresh 100 mm culture dish for 30 min in a 37 °C incubator to exclude feeding cells.
4. Collect and count floating cells, and transfer 5×10^5 cells to a fresh culture dish containing a confluent monolayer of feeding cells in 20% FBS α -MEM medium. Add the cytokine mFlt-3L (final concentration: 5 ng/mL) into the culture.
5. On day 8, gently pipette down loosely attached cells and then wash the feeding layer with 10 mL of PBS to get the maximal recovery of partially differentiated TRP2-iPSCs.
6. After harvesting cells from the coculture, centrifuge cells at $400 \times g$ for 5 min and resuspend them in 20 mL of 20% FBS α -MEM medium supplemented with mFlt-3L (5 ng/mL) and IL-7 (1 ng/mL).
7. Transfer cells into a 6-well culture plate coated with confluent OP9-feeder cells. Usually TRP2-iPSCs recovered from one 100 mm culture dish will be transferred into one well of a 6-well plate.
8. From day 10, change medium every other day (20% FBS α -MEM medium supplemented with 5 ng/mL mFlt-3L and 1 ng/mL IL-7).
9. Replace OP9 feeder cell plates every 4–6 days depending on the growth of the feeder cells.

3.4 In Vitro Analysis (Fig. 2)

1. Harvest cells at different days of coculture of TRP2-iPSCs with different lines of OP9 cells to study surface marker changes.
2. Collect cells from culture by trypsinization then washed with PBS before performing staining.
3. Blocking unspecific staining by using 24G2 antibody at 4 °C for 20 min.
4. Stain cells with a variety of fluorochrome-conjugated antibodies after blocking at 4 °C for 20 min.
5. After staining, wash cells for three times with cold PBS before flow cytometric examination.

3.5 iPSC Adoptive Transfer

In vitro-primed TRP2-iPSCs are prepared as described above.

1. After 7 days of in vitro coculture with OP9-DL1/DL4 cells, trypsinize TRP2-iPSCs, centrifuge at $400 \times g$ for 5 min, and resuspend in fresh medium.
2. Incubate harvested cells in a 37 °C incubator for 30 min to exclude differentiated and remnant feeder cells, which are firmly attached to the culture dish.
3. After incubation, collect and pellet down floating cells at $400 \times g$ for 5 min.
4. Wash cells with cold PBS for three times, and pass them through a 70 μ m nylon strainer in between washes to exclude remaining serum and cell clumps.
5. After wash, resuspend cells in cold PBS and adjust the cell density to 1.5×10^7 cells/mL (see Note 7).
6. Keep cells on ice before following with the procedure.
7. For adoptive transfer, 4–6 weeks old C57BL/6J mice are used. Before intravenous (*i.v.*) injection through the tail vein, place mice under an infrared light and further with alcohol wipes to dilate their tail veins.

3.6 Ex Vivo Analysis of CD8⁺ T Cell Cytokine Production (Fig. 3)

1. Three weeks after adoptive transfer, sacrifice the mice and collect lymphoid organs such as spleen and lymph nodes (see Note 8).
2. Harvest and isolate CD8⁺ T cells by negative selection using a magnetic beads-based kit and stimulate the CD8⁺ T cells with TRP2_{180–188} peptide since the TRP2 TCR is restricted to this unique epitope.
3. After stimulation, evaluate intracellular staining of IL-2 and IFN- γ by flow cytometry.
4. Add Brefeldin A into culture 4 h before cell harvesting.
5. Collect cells and stain surface markers as described in Subheading 3.4. Then fix cells with fixation buffer (4% paraformaldehyde).
6. Permeabilize the cells with a permeabilizing kit and perform IL-2 and IFN- γ intracellular staining with specific antibodies (Table 1) at room temperature for 20 min.
7. After staining, wash the cells in cold PBS twice and analyze the cytokine profile by flow cytometry.

3.7 Melanoma Model (Fig. 4)

Three weeks after adoptive transfer of in vitro-primed cells, inoculate B16-F10 melanoma cells subcutaneously into the right flank of C57/BL6J mice as described below (see Note 9).

1. Harvest B16-F10 cells from culture and wash them with PBS for three times.

⁷For adoptive transfer, cells need to be serum-free and clump-free to prevent pulmonary embolism and allergic response.

⁸Three weeks after adoptive transfer, a large population of CD8⁺ TCRV β 11⁺ cells were present in the lymphoid organs of mice received primed TRP2-iPSCs as compared with other controls.

⁹Tumor size is measured with a caliper and tumor volumes can be calculated as: $V = \text{long diameter} \times \text{short diameter}^2 \times 0.52$.

2. Adjust the cell density to 1×10^7 cells/mL in PBS. Keep the cells on ice during the inoculation process.
3. Shave the flank of C57/BL6J mice and inoculate subcutaneously 5×10^5 B16-F10 cells in 50 μ L of PBS into recipient mice (*see* Note 10).

3.8 Immunofluorescence, Flow Cytometric and Histological Analysis of the Tumor and Corresponding Tumor Infiltrating Lymphocytes.

At the end of the observation of tumor suppression, sacrifice the mice to check the tumor histology and TRP2-iPSCs infiltration (Fig. 4).

1. Sacrifice the mouse and surgically remove the tumor tissue.
2. Cut the tumor tissue into several pieces for different studies.
3. Fix a small piece of tumor in Neutral Formalin buffer for H&E staining and histological examination.
4. Put another small piece of tumor tissue into a cryovial and immediately submerge it into dry ice. These cryopreserved samples can be used for immunofluorescent studies.
5. Collect the rest of the tumor tissue and temporarily store it in cold PBS for flow cytometric analysis.
6. For H&E staining, the detailed protocol is as described. The histologic images were evaluated by experienced pathologists.
7. For immunofluorescent study, treat the slides with acetone after cryosection and label with an FITC- α 11 antibody before evaluation by fluorescent microscopy.
8. For flow cytometric analysis, break the tissues down mechanically into single cells and stain them with various fluorochrome-conjugated antibodies before loading onto the FACS LSR II.

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¹⁰. After tumor inoculation, mice were monitored daily for tumor growth. Tumor sizes were calculated and plotted individually, animal survivals were also documented (Fig. 5).

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Fig. 1.

Generation of TRP2-iPSCs. Murine iPSCs were overexpressed with TRP2-specific TCR by retroviral transduction. **(a)** Schematic illustration of the retroviral construct containing the TRP2-specific TCR. **(b)** GFP+ iPSCs (left) were transduced with MiDR-TRP2-TCR; GFP+ and DsRED+ double positive cells were analyzed (center) and sorted by FACS sorter (right). **(c)** Fluorescent microscopy showed the colocalized GFP and DsRED expression in transduced TRP2-iPSCs. Data are representative of three experiments

Fig. 2.

In vitro differentiation of TRP2-specific CD8⁺ lymphocytes from TRP2-iPSCs. GFP +DsRED+ iPSCs were cocultured with OP9, OP9-DL1, OP9-DL4 or OP9-DL1/DL4 cells for different time and surface markers of cultured iPSCs were evaluated by flow cytometry. (a) Expression of GFP, CD117 and CD44 on day 7 of coculture. (b) Expression of CD19, CD11b, CD25, CD44, CD4 and CD8 on day 28 of coculture. (c) Fold change of CD117⁺CD44⁺ cells after 7 days of coculture with different lines of OP9 cells. (d) Absolute cell number increases after coculture of TRP2-iPSCs with OP9-DL1/DL4 cells. Data are representative of three experiments

Fig. 3.

In vivo development of TRP2-specific CD8⁺ T cells from TRP2-iPSC after in vitro priming. TRP2-iPSCs were primed by OP9, OP9-DL1, OP9-DL4, or OP9-DL1/DL4 cells for 7 days, and then adoptively transferred into recipient mice for a 3-week in vivo development. **(a)** CD8⁺TCRV β 11⁺ T lymphocytes could be identified in a massive number from pooled lymph nodes and spleen in mice received TRP2-iPSC primed by OP9-DL1/DL4 cells. **(b)** After a 3-week in vivo development, CD8⁺ T cells pooled from lymph nodes and spleen of mice in different groups were stimulated with TRP2180–188 peptide loaded, irradiated splenocytes, and, in following days, analyzed by intracellular IL-2 and IFN- γ cytokine staining (shaded areas indicate isotype control and dark, bold lines indicate the increased fluorescent intensity). Data shown are representative of three identical experiments

Fig. 4. Tumor infiltration and suppression by CD8⁺ T cells derived from OP9-DL1/DL4-primed TRP2-iPSCs. Three weeks after adoptive transfer, mice were engrafted subcutaneously with B16 melanoma cells into the right flank. **(a)** On days 21 and 22 after tumor inoculation, mice were euthanized and tumors were resected for T cell infiltration evaluation, as well as tumor suppression. Tumor necrosis and hypocellularity could be visualized by H&E and **(b)** TRP2-specific CD8⁺TCRVβ11⁺ T lymphocytes could be visualized by immunofluorescent microscopy. **(c)** Tumor infiltrating T lymphocyte abundance was evaluated by flow cytometry. **(d)** Quantitation of tumor necrosis and **(e)** T cell infiltration of CD8⁺TCRVβ11⁺ T lymphocytes in the tumor tissue. Data represent mean (±SD) counts from six individual mice. **p* < 0.05; ***p* < 0.001. Data are representative of three experiments

Fig. 5.

Tumor management and animal protection. **(a)** Analysis of tumor mass in mice received different types of iPSCs and challenged with B16 melanoma. At Day 25 of tumor challenge, mice were euthanized and tumor sizes were evaluated. **(b)** Tumor growth Data represent mean ($\pm$ SD) tumor volume from six individual mice. One-way ANOVA test was used for statistical analyses between two groups ($*p < 0.05$). **(c)** Kaplan-Meier survival curves ($n = 6$). $*p < 0.05$; $**p < 0.001$. One-way ANOVA with Newman-Keuls Multiple comparison test. Data are representative of two experiments

Table 1

Antibodies used for flowcytometry

Name	Clone	Company
CD4	GK1.5	BioLegend
CD8	6A242	Santa Cruz
CD25	3C7	BioLegend
TCR V β 11	KT11	BioLegend
CD69	H1.2F3	BioLegend
CD44	1M7	BioLegend
CD19	6D5	BioLegend
CD62L	MEL-14	BioLegend
CD127	A7R34	BioLegend
CD11b	M1/70	BioLegend
CD117	2B8	BioLegend
IL-2	JES6-5H4	BD Pharmingen
IFN- γ	XMG1.2	BD Pharmingen