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IMMORTALIZATION OF HUMAN AND RHESUS MACAQUE PRIMARY ANTIGEN-SPECIFIC T CELLS BY RETROVIRALLY TRANSDUCED TELOMERASE REVERSE TRANSCRIPTASE

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Abstract

Human and rhesus macaque primary antigen-specific T cells derived from infected or immunized individuals or animals are a valuable material with which to study cellular immune responses against pathogens and tumors. Antigen-specific T cells can be expanded in vitro but have a finite proliferative life span. After a limited period in culture, primary T cells undergo replicative senescence and stop dividing. This restricts their applicability to short term experiments and complicates their use in adoptive immunotherapy. The proliferative life span of primary human and rhesus macaque T cells can be considerably extended by ectopically expressed human telomerase reverse transcriptase (TERT). Antigen-specific T cells transduced with TERT-expressing retroviral vectors can proliferate and expand in culture for long periods of time while maintaining their primary T cell characteristics including antigen-specific responses. Thus, TERT-immortalized T cells are an important and valuable resource for studying T cell immune responses and, potentially, for adoptive immunotherapy.

Keywords

antigen-specific T cells; TERT; telomerase; immortalization; retroviral vector; primary T cells; life span; senescence

INTRODUCTION

This unit presents a technique to immortalize human or rhesus monkey primary antigen-specific T cells by transducing them with murine leukemia virus (MuLV)-based retroviral vectors constitutively expressing human TERT. To be infectable with retroviral vectors, primary T cells have to be activated and proliferating. The first basic protocol describes how to activate resting T cells by CD3 ligation with anti-CD3 antibody bound to a plastic surface of a tissue culture plate. While the majority of human and monkey T cells can be successfully activated and proliferate vigorously after stimulation with plate-bound anti-CD3, some T cell lines and clones do not get activated or die when using this protocol. These cells can typically be activated with low concentrations of soluble anti-CD3 antibody in the presence of γ -irradiated or mitomycin C-treated human peripheral blood mononuclear cells (PBMC) as feeders. This method is described in the first alternate protocol. Human

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NOTE: Retroviral vectors expressing TERT can potentially infect humans. NIH guidelines require that all work with blood products (PBMC) and recombinant retroviruses be performed in a Biosafety Level 2 facility using BL-2 practices. Do not produce or manipulate with retroviral vectors without the proper practices, certifications and training mandated by your institution and regulatory authorities.

(but not monkey) primary T cells can also be activated by treatment with lectins such as concanavalin A, which is described in the second alternate protocol.

Retroviral vectors xlox(Δ NGFR)TERT (2) and xlox(gfp)TERT expressing TERT and a selectable marker gene (human C-terminally truncated nerve growth factor receptor, Δ NGFR, or the green fluorescent protein (GFP)) can be used to immortalize primary T cells. Transduction of activated T cells requires high titer vector preparations. The second basic protocol describes how to generate high titer TERT-retroviral vectors pseudotyped with GaLV (gibbon ape leukemia virus) or RD114 (feline leukemia virus) envelope proteins. The vectors are generated by co-transfecting packaging cells GP2-293 with TERT retroviral vector constructs and the constructs expressing either GaLV or RD114 envelope proteins. The culture media containing retroviral vector particles are harvested and used to transduce activated T cells. Two permanent clonal retroviral producer cell lines with integrated xlox(Δ NGFR)TERT and xlox(gfp)TERT vectors have been established that reproducibly generate high titers of TERT retroviral vectors simply by transfecting these cell lines with Env constructs. The elimination of vector transfection allows for a more consistent high titer retroviral vector stock production technique. The use of these cell lines to produce TERT vectors is described in the third alternate protocol.

Basic protocol 3 describes the transduction of primary T cells with TERT retroviral vectors. Vector particles are captured from the supernatant onto the bottom of a tissue culture plate by plate-bound RetroNectin, a recombinant human fibronectin fragment. Target T cells bind RetroNectin juxtaposing the cells and the vector particles and promoting the infection. Since TERT vectors express either Δ NGFR or the GFP, the efficiency of transduction can then be easily determined by measuring a frequency of either NGFR⁺ or GFP⁺ T cells in transduced T cell cultures by the flow cytometry analysis.

Since TERT-transduced T cells typically do not have a short-term proliferative advantage over untransduced cells, it is important to isolate the TERT-transduced T cells from untransduced cells soon after transduction. T cells transduced with xlox(gfp)TERT vector can be isolated by FACS, while the cells transduced with xlox(Δ NGFR)TERT can be purified either by FACS or by immunomagnetic sorting using anti-NGFR antibody. Basic protocol 4 describes the procedure for immunomagnetic sorting of NGFR⁺TERT⁺ T cells. The cells are stained with phycoerythrin (PE) - conjugated anti-NGFR antibody and labeled by anti-PE paramagnetic beads, followed by a separation on the magnetic column. Retained T cells are eluted and expanded by one of the activation methods.

Some applications of TERT-transduced immortalized T cells may require clonal T cell lines. Generation of clonal TERT-immortalized T cell lines by limiting dilution cloning is described in Basic protocol 5.

BASIC PROTOCOL 1

ACTIVATION OF PRIMARY T CELLS WITH PLATE-BOUND ANTI-CD3

ANTIBODY—This protocol describes a method to activate primary human or rhesus macaque T cells by CD3 ligation with mitogenic anti-CD3 antibody bound to a plastic surface of a tissue culture plate. The antibody is adsorbed on the plastic, and the excess of the antibody is then removed by washing. Primary T cells in the growth medium are added and cultured on the antibody-coated surface. Activation of the T cells is evaluated by observing proliferating T cell blasts.

Materials

Dulbecco's phosphate buffer saline (D-PBS, Invitrogen, Cat. No. 14190144)

Mitogenic anti-CD3 monoclonal antibody, e.g., purified NA/LE mouse anti-human CD3 ϵ antibody (Beckton-Dickinson, Cat. No. 557052). This antibody works well with both human and rhesus T lymphocytes. Store at +4°C

Polystyrene 24, 12, or 6 well tissue culture plates (Corning)

RPMI 1640 medium (Invitrogen, Cat. No. 21870-076)

Fetal bovine serum (FBS, Gemini BioProducts, Cat. No. 900-108)

Penicillin-streptomycin (Invitrogen, Cat. No. 15140-122)

Human interleukin-2 (IL-2, Roche Applied Science, Cat. No. 11147528001). Aliquot and store frozen at -20°C. Keep thawed aliquots at +4°C and use within two weeks.

T cell medium (see recipe)

Primary T cells: e.g. freshly isolated human or macaque PBMC, antigen-specific T cell line or clone

Cell counter or Trypan Blue stain (0.4%, Invitrogen, Cat. No. 15250061) for cell counting. Keep at room temperature.

Centrifuge

Incubator (37°C, 5% CO₂)

1. Prepare a fresh working solution of anti-CD3 antibody by diluting the antibody in D-PBS to a final concentration of 5 µg/ml.

Do not store anti-CD3 antibody working solution.

2. Immediately add the solution to the wells in a cell culture plate (0.3 ml for 24 well plate, 0.5 ml for 12 well plate, or 1 ml for 6 well plate).
3. Incubate the plate in the cell culture incubator at 37°C for 2 hours.

Proper humidity in the incubator chamber should be maintained to prevent the wells from drying. During plate coating, the target T cells can be prepared.

4. Spin down target T cells by centrifuging 5 min at 300 × g at room temperature. Resuspend the cells in freshly prepared T cell medium at a final concentration of 1 × 10⁶ cells/ml.

T cell lines or clones should be at the resting phase of an activation cycle (10 –14 days after previous activation). Stimulating recently activated T cells will induce cell death.

5. Remove antibody solution from the wells.
6. Wash the wells twice with D-PBS (1 ml for 24 well plate, 2 ml for 12 well plate, and 4 ml for 6 well plate).
7. Remove D-PBS and add T cell suspension in the T cell medium (1 ml for 24 well plate, 1–2 ml for 12 well plate, and 3–4 ml for 6 well plate).
8. Incubate the plates at 37°C (5% CO₂) for 48 – 72 hours.

Blasting T cells (larger, irregularly shaped cells) will be observed in 18 – 24 hours. Most human activated blasting T cells form dense clusters in 48 – 72 hours post stimulation.

9. At this step (48 – 72 hours post activation), the cells can be transduced with TERT-expressing retroviral vectors (see Basic Protocol 3).

ALTERNATE PROTOCOL 1

ACTIVATION OF PRIMARY T CELLS WITH SOLUBLE ANTI-CD3 ANTIBODY—

This is an alternative method to activate primary human or rhesus macaque T cells. Mitogenic anti-CD3 antibody is added at a low concentration directly to a suspension of target T cells, together with γ -irradiated or mitomycin C-treated human PBMC used as feeder cells. Activation is evaluated by observing proliferating T cell blasts. Some T cell lines (especially those derived from rhesus macaques) tolerate these lower doses of anti-CD3 antibody in suspension better and display more vigorous proliferation without the cell death.

Materials

Mitogenic anti-CD3 monoclonal antibody (see Materials for Basic protocol 1).

RPMI 1640 medium (Invitrogen, Cat. No. 21870-076)

Fetal bovine serum (FBS, Gemini BioProducts, Cat. No. 900-108)

Penicillin-streptomycin (Invitrogen, Cat. No. 15140-122)

Human interleukin-2 (IL-2, Roche Applied Science, Cat. No. 11147528001). Aliquot and store frozen at -20°C . Keep thawed aliquots at $+4^{\circ}\text{C}$ and use within two weeks.

T cell medium (see recipe)

Primary T cells: e.g. freshly isolated human or macaque PBMC, antigen-specific T cell line or clone

Freshly obtained human PBMC (to use as feeder cells).

Cell counter or Trypan Blue stain (0.4%, Invitrogen, Cat. No. 15250061) for cell counting. Keep at room temperature.

Centrifuge

γ -Irradiator or mitomycin C (Sigma-Aldrich, Cat. No. M4287)

Incubator (37°C , 5% CO_2)

1. Resuspend feeder PBMC in the T cell medium and irradiate at a dose of 60 Gy (6,000 Rad).
2. Spin the cells down by centrifuging 5 min at $300 \times g$ at room temperature. Resuspend irradiated PBMC in freshly prepared T cell medium at a final concentration of 2×10^6 cells/ml.

It is important to resuspend the cells in the fresh medium to remove free radicals formed during γ -irradiation.

3. If γ -irradiator is unavailable, treat feeder PBMC with mitomycin C by adding it to PBMC at a final concentration of 20 $\mu\text{g}/\text{ml}$ and incubating at 37°C for 45 min. Wash the cells three times by centrifuging 5 min at $300 \times g$ and resuspending in D-PBS or with RPMI 1640 (10% FBS). After the final wash, resuspend PBMC in freshly prepared T cell medium at a final concentration of 2×10^6 cells/ml.
4. Spin down target T cells by centrifuging 5 min at $300 \times g$. During centrifugation, add anti-CD3 antibody at a final concentration of 30 ng/ml to the γ -irradiated or mitomycin C - treated feeder PBMC suspension.

5. Resuspend target T cells in the feeder cell suspension at 5×10^5 T cells/ml (ratio of target T cells: feeder PBMC of 1:4).
6. Add the suspension to 25 cm² cell culture flasks at 5 ml per flask. Set the flasks upright and incubate at 37°C (5% CO₂) for 48 hours.
7. Add 5 ml of freshly prepared T cell medium per flask, lay the flasks down in their normal position and continue culturing the cells for additional 24 hours. If blasting and proliferating T cells are clearly noticeable at this step, the cells can be transduced with TERT retroviral vector (see Basic Protocol 3).

ALTERNATE PROTOCOL 2

ACTIVATION OF HUMAN PRIMARY T CELLS WITH CONCAVALIN A—

Primary human T cells can be easily activated by lectins such as concanavalin A (or phytohemagglutinin). This method is not recommended for rhesus macaque T cells, as they typically do not tolerate lectins and die in 2 – 4 days after a lectin has been added to the culture. Concanavalin A is added to resting primary T cells in the presence of IL-2. The cells begin dividing and grow rapidly several days later.

Materials

D-PBS

Concanavalin A (Sigma-Aldrich, Cat. No. C0412). Dissolve in PBS at a final concentration of 1 mg/ml. Aliquot and store frozen at –20°C

Polystyrene 24, 12, or 6 well tissue culture plates (Corning)

Human interleukin-2 (IL-2, Roche Applied Science, Cat. No. 11147528001). Aliquot and store frozen at –20°C. Keep thawed aliquots at +4°C and use within two weeks.

T cell medium (see recipe)

Primary T cells: e.g. freshly isolated human or macaque PBMC, antigen-specific T cell line or clone

Cell counter or Trypan Blue stain (0.4%, Invitrogen, Cat. No. 15250061) for cell counting. Keep at room temperature.

Centrifuge

Incubator (37°C, 5% CO₂)

1. Add concanavalin A to the T cell medium to reach a final concentration of 2 – 5 µg/ml.
2. Spin down primary T cells by centrifuging 5 min at 300 × g at room temperature. Resuspend the cells in concanavalin A - containing T cell medium at a final concentration of 2– 5 × 10⁵ cells/ml.

T cell lines or clones should be at the resting phase of an activation cycle (10 –14 days after previous activation). Stimulating recently activated T cells will induce cell death.

3. The cells will be blasting and begin to proliferate in 1 – 3 days.

BASIC PROTOCOL 2

GENERATION OF TERT-EXPRESSING RETROVIRAL VECTOR STOCKS—To immortalize primary T cells, they are infected with the retroviral vector expressing hTERT

cDNA. Packaging GP2-293 cells are co-transfected with retroviral vector plasmid DNA and a construct expressing either GaLV or RD114 envelope protein. The transfected cells produce pseudotyped vector particles with extended host range that are capable of infecting primary human or rhesus macaque T cells. Retroviral vector-containing cell culture medium is harvested from transfected packaging cells and used to infect target T cells.

Materials

D-PBS

Trypsin -EDTA (0.05%, Invitrogen, Cat. No. 25300054). Store at +4°C.

Cell counter or Trypan Blue stain (0.4%, Invitrogen, Cat. No. 15250061) for cell counting. Keep at room temperature.

hTERT-expressing retroviral vector construct. Two constructs expressing either Δ NGFR (2) or GFP cDNA as marker genes are available. Store frozen at -20°C

Retroviral envelope expression constructs (GaLV, or RD114). Store frozen at -20°C

Opti-MEM reduced serum medium (Invitrogen, Cat. No. 31985062). Store at +4°C

Lipofectamine 2000 transfection reagent (Invitrogen, Cat. No. 11668019). Store at +4°C

Sterile polystyrene round-bottom tubes with snap-top (12 × 75 mm, Falcon, Cat. No. 352058)

Polypropylene centrifuge tubes (15 cm, Corning, Cat. No. 430052).

10 cm poly-D-lysine coated cell culture plates (BD, Cat. No. 354469)

Packaging cell medium without antibiotics (see recipe). Store at +4°C

Packaging cell medium with penicillin-streptomycin (see recipe). Store at +4°C

1M HEPES buffer pH 7.5 (Invitrogen, Cat. No. 15630080)

GP2-293 packaging cell line (Invitrogen). Maintain the cells in packaging cell medium with penicillin-streptomycin.

GP2-293 is a derivative of human HEK 293 cell line and grows rapidly. Split the cells every two to three days 1: 10. Take great care to maintain cultures in a subconfluent (approximately 80% confluence) density. Do not allow the cells to become overgrown as they irreversibly lose transfectability. Produce a reference stock of GP2-293 by expanding the cell culture at earlier passages and freeze an ample number of vials for long term use. For best results, discard the cells that have been in a continuous culture for longer than two months, and start the new culture from the reference stock.

Centrifuge.

1. A day before transfection, plate GP2-293 cells.

- 1a** Wash a subconfluent monolayer 1 – 2 times with D-PBS, taking care not to dislodge the cells from the plate.
- 1b** Add Trypsin-EDTA and let the cells to detach for 1 min. Do not keep the cells in Trypsin longer than 2 min.
- 1c** Add 10 ml of packaging cell medium with penicillin-streptomycin to stop the trypsinization and resuspend the cells thoroughly to disperse aggregates.

1d Count the cells using Trypan blue stain and dilute the suspension to reach a final concentration of 6×10^5 viable cells/ml.

1e Add 10 ml of the cell suspension (6×10^6 cells) to a poly-D-lysine coated plate. Incubate at 37°C (5% CO₂) overnight.

- 2.** Withdraw the medium from the plate and replace it with fresh 10 ml of the packaging cell medium without antibiotics.

It is critical that antibiotics-free medium be used at this step, since Lipofectamine 2000-mediated transfection is inhibited by antibiotics.

- 3.** Prepare two mixes:

Mix A: 1.5 ml of Opti-MEM medium, 9 µg of hTERT-expressing retroviral vector plasmid DNA, 4.5 µg of the envelope expression construct plasmid DNA (GaLV, or RD114).

Both envelopes work well with both human and rhesus macaque T cells.

Mix B: 1.5 ml of Opti-MEM medium, 60 µl of Lipofectamine 2000 reagent.

- 4.** Incubate mixes A and B separately for 5 min at room temperature.

- 5.** Combine mixes A and B gently and incubate 20 min at room temperature.

Plasmid DNAs form lipid-DNA complexes during this incubation.

- 6.** Add 3 ml of the lipid/DNA complex mix drop wise to the GP2-293 cells and swirl very gently.

- 7.** Incubate the cells at 37°C (5% CO₂) for 8 hours to overnight.

- 8.** Replace the medium with 10 ml of fresh packaging cell medium with penicillin-streptomycin.

- 9.** Incubate the cells at 37°C (5% CO₂) overnight.

- 10.** Collect 48 hours vector stock. Harvest retroviral vector-containing cell culture medium by collecting the supernatant from the transfected GP2-293 cells into a 15 cm polypropylene tube.

- 11.** Add 10 ml of fresh packaging cell medium with penicillin-streptomycin to the plate and incubate overnight at 37°C (5% CO₂).

This provides an additional 10 ml of fresh retroviral vector stock (72 hours) for a repeated transduction of the target T cells next day. The titers of 72 hour-stocks are typically comparable to those harvested at 48 hours.

- 12.** Add 0.1 ml of 1M HEPES buffer pH 7.5 to 10 ml of the vector stock.

- 13.** Centrifuge buffered vector stock at $1,800 \times g$ (+4°C) for 5 min to clarify and remove cell debris.

- 14.** Carefully decant the fluid into a 15 cm polypropylene tube. The retroviral vector stock can now be used to transduce activated primary T cells.

Vector stocks can be frozen, stored at -70°C, thawed and used to transduce T cells. However, freezing and thawing causes some decrease in viral titer in vector stocks produced with GaLV or RD114 envelope proteins. The most efficient transductions are achieved with freshly prepared stocks that are used immediately.

ALTERNATE PROTOCOL 3

GENERATION OF STOCKS FROM CLONED TERT-EXPRESSING RETROVIRAL VECTOR PRODUCER CELL LINES

—To produce standard high titer TERT retroviral vector stocks, two vector producer cell lines were established by transducing packaging GP2-293 cells with xlox(Δ NGFR)TERT and xlox(gfp)TERT vectors and selecting for clones with the highest levels of retroviral vector production. Because these clones constitutively produce either vector, GP2xTERT11 (Δ NGFR vector version) and GP2xTERT-G22 (gfp vector version) without envelope proteins, they only need to be transfected with a retroviral envelope (such as GaLV, or RD114) expression construct to produce fully functional TERT vectors.

Materials

Same materials as required for Basic Protocol 2, except hTERT-expressing retroviral vector construct and GP2-293 packaging cell line

Additionally: GP2xTERT11 or GP2xTERT-G22 vector producer cell lines.

1. A day before transfection, prepare GP2xTERT11 or GP2xTERT-G22 cells as described above in Basic Protocol 2.
2. Follow step 2 in Basic Protocol 2.
3. In step 3, omit TERT retroviral vector constructs and add just the envelope expression construct (4.5 μ g) to Mix A.
4. Follow steps 4 through 13 of Basic Protocol 2.

BASIC PROTOCOL 3

TRANSDUCTION OF PRIMARY T CELLS WITH TERT RETROVIRAL VECTORS

—Primary human and rhesus macaque T cells can be transduced with TERT-expressing retroviral vectors generated as described above in Basic Protocol 2 or in the Alternate Protocol 2. Since retroviral vectors cannot infect non-dividing target cells, the absolute requirement for an efficient transduction is using freshly activated T cells that are vigorously proliferating. The optimal time of transduction following T cell activation varies depending on the specific T cell type and variability among individuals or animals. Typically, the highest transduction efficiency is achieved when the cells are transduced on day three (72 hours) post activation.

To ensure efficient infection of T cells, close contact of retroviral vector particles and target cells is promoted by RetroNectin (CH-296). RetroNectin is a recombinant protein constructed from several human fibronectin fragments, which binds both retroviral particles and the surface proteins on cells. Tissue culture plates are coated with RetroNectin, blocked and washed. Retroviral vector preparation derived as described above in Basic Protocol 2 or Alternate Protocol 2 is added and the virus is allowed to bind to RetroNectin. Target T cells are then added and are infected with the vector as they come into contact with the RetroNectin/virus-coated plastic surface.

Materials

D-PBS

RetroNectin (Takara Bio Inc., Cat. No. T100B). Store at -20°C .

RetroNectin working solution (see recipe)

Non-tissue culture treated multi-well plates (BD-Falcon, 12 well plates, Cat. No. 351143, 24 well plates, Cat. No. 351147)

Blocking solution (see recipe). Store at +4°C

Hanks balanced salt solution (HBSS, Invitrogen, Cat. No. 14170112). Store at +4°C

TERT retroviral vector stock, freshly prepared as described above in Basic Protocol 2 or Alternate Protocol 2. Avoid storing the preparation. To minimize retroviral particle inactivation and prevent vector titer loss, harvest retroviral vector-containing cell culture medium and prepare the stock at the time when RetroNectin-coated plates are being blocked by the Blocking solution.

Target T cells that were activated 72 hours before transduction as described above in Basic Protocol 1 or Alternate Protocols 1 and 2. The cells should be robustly proliferating.

Freshly prepared T cell medium. Keep at +4°C before use.

Cell counter or Trypan Blue stain (0.4%, Invitrogen, Cat. No. 15250061) for cell counting. Keep at room temperature.

Anti-human NGFR (CD271) monoclonal antibody conjugated with PE (Becton-Dickinson, Cat. No. 557196).

Refrigerated centrifuge.

Centrifuge multi-well plate holders.

Incubator (37°C, 5% CO₂)

1. Add RetroNectin working solution to the wells in the multi-well tissue culture plate (0.25 ml/well for 24 well plate, or 0.5 ml/well for 12 well plate).

Alternatively, the plates can be coated with RetroNectin at +4°C overnight. Using polystyrene non-tissue culture treated tissue culture plates is highly recommended as they are coated with RetroNectin more efficiently than tissue-culture-treated plates

2. Incubate with lid for 2 hours at a room temperature in a sterile tissue culture hood.
3. Add blocking solution (1 ml/well for 24 well plate, or 2 ml for 12 well plate).
4. Incubate for 30 min at a room temperature with lid.

During this incubation time, harvest TERT retroviral vector-containing cell culture medium from the producer cells and prepare retroviral vector stock as described above in Basic Protocol 2 or Alternate protocol 2 (steps 10 through 14).

5. Remove the blocking solution from the wells and wash them two times with HBSS, adding 1 ml/well for 24 well plate, or 2 ml/well for 12 well plate).

Take great care not to allow the wells to dry. Work fast and add the next fluid immediately after removing the previous solution. Add retroviral vector preparation to the wells immediately after the second wash.

6. Add freshly prepared retroviral stock to the wells.

The volume of the stock is not critical. You can add as much as practical for a given well capacity. Add at least enough to keep the well covered so that the centers of the wells will not dry up upon centrifugation at the next step.

7. Set the plate(s) in the multi-well plate holder(s) and centrifuge at $1,800 \times g$ for 2 hours at 30°C.

Centrifugation promotes binding of retroviral vector particles to the plate-bound RetroNectin. Make sure that the temperature is kept stably at 30–32°C as this maximizes binding of the virus to the RetroNectin and minimizes the thermal inactivation of retroviral particles. Do this in a sealed centrifuge and decontaminate any spilled material. Alternatively, you may incubate the plate(s) at 30–32°C without centrifugation for 6 hours.

8. While binding the vector to RetroNectin-coated plates, prepare target T cells. Spin down the cells and resuspend in the freshly prepared T cell medium supplemented with IL-2 (see recipe). Count the cells and dilute the suspension to a final concentration of 1×10^6 cells/ml.
9. Remove the fluid from the RetroNectin/vector-coated wells and wash once with the blocking solution (add 1 ml/well for a 24 well plate, or 2 ml/well for a 12 well plate).

Washing removes contaminating cellular proteins that are often present in the retroviral stock and can inhibit retroviral infection. It is extremely important to work fast and avoid well drying to prevent retrovirus inactivation.

10. Add target T cells to the wells (1 ml/well for a 24 well plate, or 1 – 2 ml/well for a 12 well plate). Set the plate(s) in the multi-well plate holder(s) and centrifuge at $300 \times g$ for 30 – 40 min at 30°C.

Centrifugation speeds up binding of the cells to the RetroNectin and promotes cellular contacts with RetroNectin-bound retroviral vector particles, thus enhancing the transduction efficiency. Keep the temperature at 30–32°C as this is optimal for retroviral infection and decreases virus inactivation.

11. Stop the centrifuge, remove the plate(s) from the holders and incubate at 37°C (5% CO₂) overnight.
12. (Optional) To further increase transduction efficiency, repeat the transduction of the cells the next day with the freshly collected 72 hours retroviral vector stock (steps 1 – 11).
13. To determine the efficiency of transduction, perform standard flow cytometry analysis of transduced T cell populations. In the T cells transduced with xlox(gfp)/TERT vector, measure GFP expression directly. If the cells were transduced with xlox(ΔNGFR)/TERT, stain with anti-human NGFR-PE conjugated antibody. The cells can be also co-stained with desired surface marker-specific antibodies to lymphocyte markers for phenotype analysis.

Allow at least 48 hours after the transduction with TERT vectors before performing the flow cytometry analysis. This time is necessary to complete retroviral DNA integration and to express TERT and marker proteins (ΔNGFR, or GFP). The transduction efficiency critically depends on the proliferation rate of the T cells and on type of T cells being transduced.

14. Proceed with purification of transduced T cells by immunomagnetic sorting or FACS.

BASIC PROTOCOL 4

ISOLATION OF TERT-TRANSDUCE T CELLS BY IMMUNOMAGNETIC

SORTING—Primary T cells transduced with xlox(Δ NGFR)TERT retroviral vector express human C -terminally truncated NGFR on the surface and can be rapidly and efficiently purified by immunomagnetic sorting. The cells are stained with PE-conjugated anti-human NGFR (CD271) monoclonal antibody, washed, and labeled with anti-PE paramagnetic microbeads. NGFR⁺ TERT⁺ cells are then separated from the untransduced NGFR[−] cells on a magnetic separation column, eluted, and cultured to establish TERT-immortalized T cell lines.

Materials

TERT-transduced primary T cell culture

Feeder cells (normal human PBMC)

Cell counter or Trypan Blue stain (0.4%, Invitrogen, Cat. No. 15250061) for cell counting. Keep at room temperature.

Polypropylene tubes (15 cm, Corning, Cat. No. 430052).

Magnetic sorting buffer (see recipe). Store at +4°C. Keep on ice when using.

Anti-human NGFR (CD271) monoclonal antibody conjugated with PE (Becton-Dickinson, Cat. No. 557196).

Anti-PE paramagnetic microbeads (Miltenyi Biotec, Cat. No. 130-048-801). Store at +4°C

LS MACS separation columns (Miltenyi Biotec, Cat. No. 130-042-401). Store at room temperature.

MidiMACS Separation Unit (Miltenyi Biotec, Cat. No. 130-042-302). Pre-chill at +4°C before cell separation.

MACS MultiStand (Miltenyi Biotec, Cat. No. 130-042-303). Pre-chill at +4°C before cell separation).

Tissue culture plates or flasks

T cell medium containing IL-2.

Refrigerated centrifuge

1. Count TERT-transduced T cells. Spin the cells down in a 15 ml centrifuge tube at $300 \times g$ (4°C) for 5 min, discard the supernatant and resuspend the cells in ice-cold magnetic sorting buffer (use 0.4 ml of the buffer if the total number of cells is 1×10^7 cells or less, or 0.8 ml if the number of the cells is $0.5 - 1 \times 10^8$).
2. Place the tube on ice.
3. Add PE-conjugated anti-NGFR antibody (20 μ l per 1×10^7 cells or less, 40 μ l per $0.5 - 1 \times 10^8$ cells), mix gently by tapping the tube.
4. Transfer the tube at +4°C and incubate for 15 – 20 min.
5. Add 5 ml of ice-cold magnetic sorting buffer and gently mix by pipetting.
6. Spin down in pre-chilled centrifuge at $300 \times g$ (4°C) for 5 min, discard the supernatant and resuspend the cells in the original volume of ice-cold magnetic sorting buffer. Keep the tube on ice.

7. Add anti-PE paramagnetic beads (20 μ l per 1×10^7 cells or less, 40 μ l per $0.5\text{--}1 \times 10^8$ cells). Mix gently by tapping.
8. Transfer the tube at $+4^\circ\text{C}$ and incubate for 15 – 20 min.
9. Add 5 ml of ice-cold magnetic sorting buffer and gently mix by pipetting.
10. Spin down in pre-chilled centrifuge at $300 \times g$ (4°C) for 5 min, discard the supernatant containing unbound beads and resuspend the cells in 1 ml of ice-cold magnetic sorting buffer. Keep the tube on ice.

While centrifuging, perform steps 11–12 below.

11. Attach LS magnetic separation column to a MidiMACS Separation Unit and stick the unit to the MACS MultiStand
12. Equilibrate the column by loading 3 ml of ice-cold magnetic sorting buffer and allowing it to flow through.
13. Load 1 ml of labeled cell suspension and allow it to flow through.
14. Remove the unbound cells from the column by washing four times with 3 ml of magnetic sorting buffer, each time allowing it to drain completely.
15. Detach the column from the separation unit and place it on top of a clean 15 ml centrifuge tube.
16. To elute NGFR-positive cells, add 5 ml of magnetic sorting buffer or T cell medium to the column and expel the solution containing the cells into the tube using the column plunger.
17. Prepare γ -irradiated or mitomycin C-treated feeder PBMC as described in the Alternate protocol 1 (steps 1 – 3).
18. Add 5 ml of feeder cell suspension to the eluted NGFR-positive T cells and spin down at $300 \times g$ for 5 min at room temperature.

If the number of the eluted T cells is small, spinning them down alone may result in the cell loss. To prevent this, add feeder cells as a carrier to the eluted T cells and spin them down together.

19. Discard the supernatant and resuspend the sorted T cells/feeder cells mixture in 5 ml of a fresh T cell medium containing IL-2 (see recipe). Add mitogenic anti-CD3 antibody (see Basic Protocol 1 and Alternate Protocol 1) to a final concentration of 30 – 50 ng/ml.
20. Add the cell suspension to a 25 cm^2 cell culture flask, set it upright and culture at 37°C (5% CO_2) for 48 hours.
21. Add 5 ml of fresh T cell medium containing IL-2, lay the flask down into the normal position and culture at 37°C (5% CO_2).
22. Inspect the culture under the microscope. Sorted NGFR⁺TERT⁺ T cells should begin to proliferate vigorously 48 hours post sorting/activation. Feeder cells begin dying on day one post irradiation and are expected to be completely eliminated in several days. Isolated proliferating T cells can be further expanded into the cell lines and can be frozen for storage, used in the experiments or cloned as described below in Basic protocol 5.

BASIC PROTOCOL 5

GENERATION OF CLONAL TERT-IMMORTALIZED T CELL LINES BY LIMITING

DILUTION—To create stable and homogenous source of immortalized T cells, generation of clonal T cell lines from TERT-transduced T cells is advised. Purified TERT-transduced T cells are cloned by limiting dilution. Serial dilutions of T cell population are mixed with γ -irradiated or mitomycin C-treated feeder human PBMC in the presence of IL-2 and mitogenic anti-CD3 antibody. The mixes are then plated in 96-well plates. Isolated clones are expanded into cell lines.

Materials

TERT-transduced T cell line prepared by immunomagnetic (see Basic Protocol 4) or FACS sorting

Cell counter or Trypan Blue stain (0.4%, Invitrogen, Cat. No. 15250061) for cell counting. Keep at room temperature.

Freshly prepared T cell cloning medium (see recipe)

Multi-channel pipette

Sterile plastic 50 ml reagent reservoirs (Corning, Cat. No. 4870)

Polystyrene round bottom tissue culture 96 well plates (Costar, Cat. No. 3799)

Sterile polypropylene 50 ml centrifuge tubes (Corning, Cat. No. 430290)

Incubator (37°C, 5% CO₂)

1. Count the cells in the TERT-transduced T cell suspension.
2. Prepare feeder cells by γ -irradiating freshly obtained human PBMC (if γ -irradiator is unavailable, treat PBMC with mitomycin C, see Alternate Protocol 1).
3. Use feeder cells to prepare T cell cloning medium (see recipe).
4. In a 50 ml tube, prepare a starting dilution of the T cells to be cloned in 40 ml of T cell cloning medium. Starting T cell concentration depends on the plating efficiency of the T cells and may vary.

Typically, the starting dilution of the T cells is 50 cells per well (50 cells in 200 μ l, or 250 cells/ml). After adding an aliquot of the T cells, mix gently but thoroughly by repeated pipetting.

5. Distribute T cell cloning medium into nine 50 ml centrifuge tubes at 20 ml/tube.
6. Serially dilute the starting dilution among the nine 50 ml tube beginning with adding 20 ml of the starting dilution into tube one and mixing well by pipetting. 20 ml of this higher dilution is then dispensed into tube two. Repeat to produce the remaining dilutions. Change the pipette each time before preparing a new dilution in the series.

If started with 50 cells per well, concentrations of 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4, 0.2, and 0.1 cells per well will be obtained.

7. Plate each dilution into a 96 well plate, changing the reservoir and tips for each dilution. Alternatively, use the same reservoir and tips and plate from high to lower dilutions.

Pour the fluid into the plastic reservoir and distribute into the wells at 200 μ l per well by using multi-channel pipette.

8. Place the plates into the incubator and culture at 37°C (5% CO₂) for one week.
Take great care to maintain proper humidity in the incubator, to prevent drying the wells.
9. On day 7, withdraw ½ (100 µl) of the medium from the wells by using multi-channel pipette.
10. Prepare fresh T cell cloning medium without the feeder PBMC and anti-CD3 antibody. Make sure to supplement the medium with IL-2 at double concentration (200 IU/ml).
11. Distribute 100 µl of the medium per well and culture for one additional week.
The time when the growing clones of the T cells become visible varies and depends on the T cell type and source. Normally, the clones become visible on day 10 and are ready to be picked on day 14.
12. Select the plates with the 30% or less positive wells. Mark the wells with the clones selected for picking on the plate lid.
13. Prepare fresh complete T cell cloning medium and distribute into the wells of a 24 well tissue culture plate at 1 ml per well.
14. Resuspend each selected clone and add to 1 ml of the T cell cloning medium in the 24 well plate.
15. Incubate the plate for several days until cloned T cells in the wells proliferate and start dividing rapidly. Dilute these cultures in larger volumes of T cell medium containing IL-2 at 100 IU/ml. Continue expanding the cultures until they cease rapid proliferation.
16. At this step, clonal cell lines can be expanded further as described above in Basic protocol 1 and in Alternate protocols 1 and 2.

REAGENTS AND SOLUTIONS

T cell medium

RPMI 1640 supplemented with 10% FBS, glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and IL-2 (100 IU/ml). Store the medium without IL-2 at +4°C. Add IL-2 to the required volume of the medium just before using.

xlox(ΔNGFR) TERT and xlox(gfp)TERT

retroviral vectors expressing hTERT and marker genes

Packaging cell medium without antibiotics

supplement DMEM (high glucose, Invitrogen, Cat. No. 10569010) medium with 10% FBS. Store at +4°C.

Packaging cell medium with penicillin-streptomycin

supplement DMEM (high glucose) medium with 10% FBS and add penicillin-streptomycin mixture (Invitrogen, Cat. No. 15140-122) to a final concentrations of penicillin of 100 U/ml and streptomycin of 100 µg/ml. Store at +4°C.

Blocking solution

prepare 2% solution of bovine serum albumin (Sigma-Aldrich, Cat. No. A2153) in D-PBS. Sterilize by filtration through 0.45 µm pore-membrane and store at +4°C.

RetroNectin working solution

dissolve RetroNectin in D-PBS and dilute with D-PBS to a final concentration of 20 µg/ml. Do not store, use immediately to coat plates.

Magnetic sorting buffer

prepare 0.5% solution of bovine serum albumin (Sigma-Aldrich, Cat. No. A2153) in D-PBS. Add EDTA to a final concentration of 2 mM. Sterilize by filtration through 0.45 µm pore-membrane and store at +4°C.

T cell cloning medium

Add the following to RPMI 1640 medium: glutamine to a final concentration of 2 mM, FBS (20%), penicillin-streptomycin (penicillin at 100 U/ml and streptomycin at 100 µg/ml), IL-2 to a final concentration of 100 IU/ml, feeder cells (γ-irradiated or mitomycin C-treated human PBMC, see Alternate Protocol 1) to a final concentration of 5×10^5 cells/ml. Always prepare fresh, do not store. Immediately before use, add mitogenic anti-CD3 antibody (see Basic Protocol 1) to a final concentration of 50 ng/ml.

COMMENTARY

Background Information

T lymphocytes continuously differentiate from hematopoietic stem cells to acquire specific immune functions. Unlike most other somatic cells, fully differentiated T lymphocytes do not lose the ability to divide and can be stimulated by antigens or non-specific stimuli to proliferate and expand in culture or *in vivo*. Over time, proliferating T cells ultimately enter a phase of replicative senescence after reaching a certain number of population doublings (the Hayflick limit). During this phase the cells stop dividing and can survive in culture without proliferation in the presence of IL-2 for certain periods of time.

As in the other mammalian cells, the chromosome ends in T cells contain telomeres, which are specialized structures that consist of repetitive DNA sequences of varying length (telomeric repeats). Telomeres are associated with several proteins that form a complex, which protects chromosomes from fusion and chromosome ends from being improperly recognized as DNA break/damage signals (15, 32–33). With each DNA replication cycle, telomeres are shortened (12) and ultimately reach a critical length, at which *p53*-mediated growth arrest mechanism is induced and cell proliferation is ceased (33). While young T cells have longer telomeres, telomere length in aged and senescing T cells is much shorter. Telomere shortening is thought to function as a molecular clock, which imposes a limit on proliferative capacity and population expansion of T cells and serves as a signal for the onset of replicative senescence.

Shortened telomeres in T cells can be stabilized or restored by TERT, which is a specialized reverse transcriptase capable of extending telomere repeats by synthesizing DNA strand on the template of specialized telomerase RNA. TERT is exclusively expressed in the cells that normally are capable of long term proliferation (e.g., embryonic stem cells), but not in normal differentiated somatic cells except for lymphocytes (5, 7, 9, 16, 23).

TERT activity in normal human T cells is tightly regulated. Circulating quiescent T cells do not express TERT; however, TERT activity is sharply elevated in activated human T cells (14, 17, 36–39) and may play a role in supporting the survival, proliferation, and expansion of antigen-responding T cells *in vivo*. Thus, T lymphocytes are unique among differentiated somatic cell types in terms of their ability to respond to specific or nonspecific stimuli by proliferation and expansion, accompanied by TERT upregulation. In long-term T cell

cultures, the levels of TERT induced upon initial T cell activation reach a peak at around 3 to 5 days and then decline over the following two weeks (11, 38). While dramatic increase of TERT activity occurs upon primary stimulation of T lymphocytes, subsequent stimulation cycles induce less TERT expression, which eventually becomes nearly undetectable as the cells progress towards senescence. Endogenous telomerase activity levels are not sufficient to completely block telomere loss and to extend T cell proliferative life span beyond their natural Hayflick limit. Human T cells expressing dominant-negative TERT have a decreased proliferative life span in culture and develop cytogenetic abnormalities such as chromosome fusions and the lack of telomeric DNA at the chromosome ends (30). The inverse correlation between TERT activity levels and replicative history suggests that TERT expression in human T cells is limited and insufficient to sustain extended periods of proliferation.

The proliferative life span of human primary antigen-reactive T cells in culture can be significantly extended by introducing ectopically expressed TERT gene. Previous work demonstrated that virus-specific human T cells isolated and cloned from healthy donors and transduced with a retroviral vector constitutively expressing TERT had considerably extended longevity in culture and maintained antigen-specific reactivity (18, 31). TERT overexpression can be used to increase the survival of the primary T cells and to protect them from apoptosis. The longevity of human CD4⁺ helper type 1 or 2 T cells in culture was increased by TERT gene overexpression (27). T cells ectopically expressing TERT were found to expand more vigorously than untransduced cells of the same replicative age. Importantly, in comparison to the untransduced T cells, TERT-transduced cells expressed elevated levels of anti-apoptotic Bcl2 protein, contained lower active caspase-3 and were resistant to telomere DNA-damaging oxidative stress. Thus, in addition to protecting cultured T cells from replicative senescence, conferring resistance to apoptosis afforded by ectopically expressed TERT could further increase their survival.

In addition to displaying proper antigen recognition and effector functions *in vitro*, TERT-overexpressing human tumor-reactive T cells were found to maintain full anti-tumor reactivity in an *in vivo* murine cancer model (34). Adoptively transferred TERT-transduced influenza virus-specific human CTL clones inhibited the growth and caused regression of human melanoma tumors induced by tumor cells marked with an influenza virus epitope with the same efficiency as observed with untransduced CTL clone, suggesting that human TERT-transduced T cells can maintain antigenic specificity and a full set of effector functions *in vivo*. Thus, human T cells overexpressing TERT can be potentially used for adoptive immunotherapy.

While considerable interest exists in establishing the ways to extend the life span of human primary T cells, primary T lymphocytes derived from non-human primates (NHP) are another important target. NHP provide an attractive model for research of immune responses to lentiviral infections. Rhesus monkeys (RM) infected with simian immunodeficiency virus (SIV) are widely used to model the pathology of human AIDS and cellular immune responses to HIV-1. Similar to human T cells, *in vitro*-expanded RM T lymphocytes enter a phase of replicative senescence and have a limited proliferative life span in culture. TERT-immortalized RM antigen-specific T cells might provide an invaluable source for studies of SIV immune responses and for experimental adoptive immunotherapy. Effector memory CD8⁺ T cell clones specific for immunodominant SIV *Gag* and *Tat* epitopes (26) were derived from Mamu A*01 RMs and transduced with a retroviral vector expressing human TERT (2). Transduced cell lines exhibited substantially better survival in long term *in vitro* cultures in comparison to their untransduced parental clones, responded specifically to antigenic stimulation, and displayed comparable proliferation rates, cytokine expression profiles and cytolytic activities. Furthermore, these T cell lines constitutively expressing TERT were shown to respond to the SIV-infected autologous CD4⁺ target T cells by IFN- γ .

production and degranulation and were able to significantly suppress SIV replication (28). Thus, similar to human T cells, primary NHP antigen-specific T cells can be successfully immortalized by human TERT. Immortalized NHP T cells retain full potential for antigen recognition and effector functions and may prove to be a valuable tool for developing experimental immunotherapy protocols.

Retroviral vector-mediated TERT transduction can be used to simultaneously genetically tag and immortalize human T cells responding to a specific antigen. Infection with MuLV-derived retroviral vectors expressing human TERT allows selective capture and immortalizing of human antigen-specific T cells from a complex cell population such as blood (3–4). Such vectors can only integrate in dividing cells (24, 29), thus being able to selectively transduce only those T cells in a population that are specifically activated and dividing. Human peripheral blood mononuclear cells (PBMC) stimulated with alloantigen and infected with TERT-expressing vectors gave rise to CD8⁺ antigen-specific T cell lines with considerably extended proliferative life span. The lines were generated by passaging the transduced cells for extended periods of time with no other selection steps involved, showing that antigen-responding T cells could be selectively captured, genetically marked and immortalized by TERT vector transduction. TERT-immortalized CD8⁺ allospecific T cell lines maintained IL-2-dependent growth, specifically responded to cognate alloantigen by proliferation and production of cytokines, and exhibited antigen-specific cytolytic activity. Thus, engineering of human and NHP T cells to overexpress TERT can provide significant life span extension to valuable antigen-specific T cell lines and clones without the loss of their primary T cell characteristics, providing cellular immunologists with lasting source of well-characterized T cells. In addition, protection from replicative senescence *in vivo*, afforded by TERT overexpression, may make immortalized T cells an attractive tool for adoptive transfer-based immunotherapy.

Are TERT-transduced primary T cells truly immortalized? We and the others were able to maintain human and RM TERT-transduced antigen-specific T cell lines and clones in culture for periods over one year without the loss of specific T cell functions, which far exceeds the normal proliferative life span of primary human and NHP T cells (2, 4, 8, 28). However, it remains to be established that TERT-transduced T cell lines and clones are truly immortal, rather than protected from senescence for extended but still finite period of time. Long term observations of TERT-immortalized T cell lines are necessary to determine their actual longevity *in vitro*.

Genes other than TERT have been used to extend the life span of human primary T cells. T cells transduced with retroviral vectors expressing viral oncogenes such as HTLV-I Tax (4) or infected with Herpesvirus saimiri that expresses STP-C488 oncogene (1, 10, 13, 20–22) have extended longevity in culture. Tax-transduced human allospecific and Hepatitis B surface antigen-specific T cells could be cultured for many months without losing their antigen-specific reactivity (4). The cells were dependent on IL-2 to grow and were maintaining proper activation-rest cycles in culture when stimulated with their cognate antigens. However, since both Tax and STP-C488 are oncogenes capable of transforming human cells, the possibility of a malignant transformation of Tax- or STP-C488-transduced primary T cells upon sufficiently extended time in culture is real. Since TERT does not transform primary mammalian cells, using TERT to immortalize human and macaque primary T lymphocytes is conceivably a safer approach.

Since retroviral vectors permanently integrate into the genome of a host cell and may activate the expression of cellular oncogenes, the use of retroviral vectors to deliver TERT gene in the primary T cells may not be appropriate for certain clinical applications of engineered T cells. Alternative approaches to induce endogenous TERT expression in

primary T cells at the levels sufficient to extend their proliferative life span might prove possible. TERT expression can be upregulated in the primary T cells by cytokines implicated in maintenance, survival, and homeostatic control, such as IL-7 and IL-15 (19, 25, 35, 40). Retroviral transduction of a primary human CD8⁺ T cell clone with the IL-15 gene generated a cell line that constitutively expressed TERT and exhibited external cytokine-independent growth for more than a year while preserving the capability for antigen-specific activation, suggesting that IL-15 autocrine loop alone is sufficient to ensure TERT-dependent extension of the T cell life span (19). Thus, IL-7 and IL-15 can increase the longevity of the T cells by supporting stable and sustainable telomere length through upregulated TERT expression, and can potentially be used as an alternative to direct TERT-induced immortalization. Nuclear factor of activated T cells (NFAT1) is directly involved in transcriptional activation of TERT expression (6). TERT promoter has putative NFAT1 binding sites and is activated by overexpressed NFAT1. It remains to be seen whether constitutive overexpression of NFAT1 in primary T cells can immortalize them without the loss of normal immune functions.

Critical Parameters and Troubleshooting

The most critical single element required for the success of the retroviral vector-mediated TERT transduction protocols described here is the robustness of proliferation of the target T cells. Mo-MuLV-derived retroviral vectors cannot infect the cells that are not dividing. Therefore, the efficiency of transduction of primary T cells is linearly dependent on how fast activated cells proliferate. For any particular target T cell type, it is important to establish the conditions for T cell activation resulting in the most vigorous proliferation activity. Slower dividing T cells produce lower transduction efficiency and are more difficult to purify and establish permanent cell lines from, while it is much easier to efficiently transduce and isolate the T cells that grow vigorously.

In addition to activation by mitogenic anti-CD3 antibodies and lectins described here, antigen-specific T cells can be stimulated with their cognate antigens. The protocol for the most efficient stimulation with an antigen depends on the T cell source, type, and the nature of the antigen and should be established individually. The time between stimulation and TERT retroviral vector transduction is another critically important factor, which depends on the way the T cells were stimulated. We found that typically days three through four post stimulation with plate-bound or soluble anti-CD3 antibody, or with concanavalin A (Basic Protocol 1 and Alternate protocols 1 and 2) is an optimal opportunity window to efficiently infect activated primary T cells with retroviral vectors. However, this time may vary depending on the specific source of T cells. Investigators are advised to establish the optimal transduction time in their specific systems experimentally, especially when the T cells must be stimulated with antigens.

Traditionally, retroviral vectors were pseudotyped with vesicular stomatitis virus (VSV) G protein, as this creates vectors with the broadest host range that are capable of infecting a variety of mammalian cells. However, we and the others have found that while VSV-G-pseudotyped vectors efficiently transduce human permanent T cell lines such as Jurkat or SupT1, they are significantly less infectious for activated primary human and rhesus monkey T cells. The envelope proteins from two other retroviruses, GaLV and feline leukemia virus RD114 are readily incorporated into MuLV vector particles and convey to them a superior capacity to infect primary human or NHP T cells. We have found that using either envelope to produce TERT-expressing vectors xlox(Δ NGFR)TERT and xlox(gfp)TERT results in equally good transduction efficiency in both human and RM T cells, both being superior over VSV-G.

Obtaining high retroviral vector titer from transfected GP2-293 or xlox vector producer cells is very important for achieving good transduction efficiency. The key point in generating high titer retroviral vector stocks is using rapidly dividing packaging/producer cells that have been cultured at a lower density for a short term. The cells should always be maintained in a subconfluent state (less than 80% confluent) and must not be allowed to overgrow, as this dramatically and permanently decreases their transfectability. Transduction of primary T cells with higher titer TERT vectors normally results in higher percentages of transduced cells, which require less time and efforts to purify and expand. For instance, when using xlox(Δ NGFR)TERT vector, it is possible to obtain 95 – 100% pure transduced T cells with a single round of immunomagnetic purification (as described in Basic protocol 4). Pure transduced cells can normally also be isolated from the populations with only 1 – 5% of positive cells; however, this typically requires two to three rounds of immunomagnetic purification to obtain high purity populations of transduced T cells..

When performing RetroNectin-mediated retroviral transduction of T cells, it is critically important to use freshly prepared retroviral vector stocks. Although freezing and storing them is possible, the highest titers and the best transduction efficiencies are usually achieved by using freshly harvested vector-containing supernatants. At each step of the protocol, it is absolutely necessary to keep wells in a cell culture plate wet and never allow them to dry in the hood, as this may denature RetroNectin and inactivate bound retroviral vector particles.

Importantly, sufficient time (typically, at least 48 hours) should be allowed before proceeding to the flow cytometry analysis of transduced T cells. It requires approximately 9 – 11 hours for a retrovirus to complete infection of the target cells and integration into the host DNA. Marker genes will need to start expressing from the integrated vector cassette and accumulate in the infected T cells in the amounts sufficient to be detectable by the flow cytometry. Therefore, attempts to analyze the transduced cells too early may lead to a false-negative result.

TERT expression in the primary T cells does not seem to increase their proliferation rate as compared to the untransduced cells. Accordingly, transduced T cells do not tend to accumulate and overgrow the untransduced counterparts in the short term, and can be lost from cultures upon prolonged passaging and expansion if the transduction efficiency is not very high. Investigators are recommended to purify and expand TERT-transduced T cells as soon as possible after the successful transduction with a TERT vector was established by the flow cytometry analysis.

Since there is no available quick assay to verify that the transduced T cells are immortalized, prolonged culture of TERT-transduced T cell lines remains the only way. Typically, TERT-transduced T cell lines easily expand and grow in culture past the normal expected time limits for primary T cells. By now, we maintained several TERT-transduced antigen-specific rhesus monkey T cell clones in a continuous culture for several years, thus clearly exceeding normal primary T cell life span. However, it is important to remember that, similarly to their unmodified counterparts, TERT-transduced T cells maintain all characteristics of normal primary T lymphocytes and require proper maintenance, such as periodic stimulation and expansion alternating with rest periods, maintaining the cultures at densities not exceeding $1 - 2 \times 10^6$ cells/ml, and adding IL-2 every 2 to 3 days. Since valuable T cell clones (immortalized or not) may change after prolonged periods in culture, sufficient number of vials with freshly obtained and expanded TERT-transduced T cell lines should be frozen and stored in liquid nitrogen as soon as possible to create a lasting source of T cells.

Anticipated Results

Typical transduction of human and rhesus macaque primary T cells with TERT retroviral vectors results in 5 – 40% of positive cells detectable by the flow cytometry 48 hours post transduction, although lower or higher efficiencies might occur, depending of the target T cell type and proliferation rate. When using vectors expressing Δ NGFR marker gene, one to three rounds of immunomagnetic sorting are usually sufficient to obtain pure transduced populations of T cells. Purified TERT-transduced T cells can be maintained in culture for very long periods of time with periodic re-stimulation followed by an expansion in the presence of IL-2.

Time Considerations

The time required to obtain a TERT-immortalized T cell line varies depending on the proliferation rate of a starting T cell population, T cell type and source, and the efficiency of transduction with TERT retroviral vector. Normally, setting up pre-transduction activation cultures of primary T cells takes several hours. Activation of the cells in culture requires two to three days. Co-transfection of packaging cell lines with TERT vectors and envelope constructs can be completed in one hour and vector stocks can be prepared in 48 and 72 hours post transfection. With proper planning, fresh retroviral vector stocks are obtained at a day the T cells are ready for transduction.

Transduction of the T cells with TERT vectors can be completed easily within one working day. The procedure requires approximately 5.5 – 6 hours. The transduced T cells can be analyzed for expression of Δ NGFR or GFP 48 hours post transduction. Sorting positive cells requires one working day, and expansion of sorted transduced T cells may take from several days to one or two weeks if the initial number of transduced cells was low. Altogether, three to four weeks are required to generate TERT-transduced T cell lines.

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