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Isolation, activation, and retroviral transduction of primary T cells from murine splenocytes

In 2 collections

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ABSTRACT

This protocol outlines the steps for retroviral transduction of activated primary T cells isolated from spleen of donor mice.

1 Day 1: Plating Platinum-E (Plat-E) retroviral packaging cell lines

1.1 Coat wells of 6 well plates with 1 mL of poly-L-lysine. Let sit at RT for 10 mins.

1.2 Aspirate supernatant from early passage # Plat-E cells (Cell Biolabs RV-101)

1.3 Add 5 mL 0.05% Trypsin-EDTA to detach cells. Incubate at 37'C for 2-3 mins

1.4 Quench reaction by adding 5 mL DMEM + 10% FBS

1.5 Transfer to a 15 mL conical, pipet up and down and vortex briefly, and take 10 uL to count. Centrifuge at 1200 rpm for 7 mins.

1.6 Aspirate supernatant from cell pellets. Resuspend to 8 x 10^5 cells/mL with DMEM + 10% FBS.

1.7 Aspirate poly-L-lysine from 6-well plates. Add 1 mL (8 x 105 cells) of the cell suspension to each well. Add an additional 2 mL of DMEM + 10% FBS to each well (total volume is 3 mL/well).

1.8 Let cells grow overnight in incubator set to 37'C, 5% CO2.

2 Day 2: Transfecting PlatE cells

2.1 Check cells to make sure they are 70-80% confluent.

2.2 Prepare transfection reagents in the following order in 2 separate 15mL conical tubes:

2.3 Plasmid DNA tube - Per 1x reaction (scale up according to how many wells you need to transfect with the same CAR T construct): 10ug DNA in 250 uL pre-warmed OPTI-MEM, vortex.

2.4 Lipofectamine tube - Per 1x reaction (scale up according to how many wells you need to transfect with the same CAR T construct): 20 uL Lipofectamine 2000 (Invitrogen 11668019) in 250 uL pre-warmed OPTI-MEM. DO NOT VORTEX, instead add lipid to the center of the tube and mix by pipetting up and down. Incubate for 5 minutes at room temperature.

2.5 Transfer the DNA tube contents to the lipofectamine tube, and mix by pipetting up and down.

2.6 Incubate at room temperature for 20 minutes.

2.7 During the 20min incubation, aspirate supernatant from Plat-E cells in 6-well plates, wash cells with 2 mL of pre-warmed OPTI-MEM, swirl then aspirate. Add 1.5mL OPTI-MEM to each well.

2.8 After the 20 min incubation, dispense 500 uL of the Lipofectamine/DNA mix dropwise to cells in respective wells and incubate for 4 hours at 37'C, 5% CO2.

2.9 After 4 hours, aspirate media and add 3.5 mL fresh IMDM + 10% FBS and incubate at 37'C, 5% CO2 for 48 hours.

3 Day 3: Isolation and activation of primary T cells from splenocytes

3.1 Prepare the following before you start: Prepare ice buckets, thaw 1 mL FBS aliquot on ice, place surgery tools in sterilizer, prepare absorbent sheets, aliquot 15 mL RPMI into 50 mL tubes (two spleens/tube), prepare 100um nylon mesh filters, petri dishes, 3 mL syringes in tissue culture hood

3.2 Sacrifice donor mice by CO2 asphyxiation and cervical dislocation. Lay mice on its right side, spray with 70% ethanol, and surgically remove spleen using sterile scissors and forceps. Transfer spleen in 50mL tube containing RPMI media. Keep on ice. Put carcass in bag and dispose in necropsy freezer.

3.3 Working inside a tissue culture hood, smash spleens with the syringe plunger through the mesh filter in the petri dish. Pour the 15 mL RPMI through the filter to flush. Collect cells in 50 mL conical tube.

3.4 Spin cells at 300xg for 5 min 4'C.

3.5 Decant supernatant, and resuspend cells in 7.5 mL ACK lysis buffer

3.6 Incubate at room temperature for 2 minutes. Stop reaction with 7.5 mL RPMI + 10% FBS. Spin cells at 300xg for 5 min 4'C.

3.7 Wash cells with 20 mL RPMI twice, count cells and take a small aliquot for Flow cytometry staining before 2nd spin

3.8 Isolate T cells using Dynabeads Untouched mouse T cell kit (Life Technologies Cat. # 11413D) following manufacturer's protocol. After T cell isolation, take a small aliquot for Flow cytometry staining to assess purity of T cell preparation.

3.9 Activate purified T cells using anti-CD3/anti-CD28 Dynabeads (Life Technologies Cat. # 11452D) for 24 hours per manufacturer's instructions in complete IMDM media supplemented with 15U/mL IL-2 (Gibco PMC0025).

3.10 Assess viability and purity by Flow cytometry using viability dye, anti-CD3 (or anti-TCR Cβ), anti-CD8, and anti-CD4 antibodies.

3.11 Coat 24-well non-TC treated plates with 15ug/mL Retronectin (Takara T100B), prepared by diluting 1mg/mL stock to 15 ug/mL with PBS. Dispense 500 uL/ well, seal plates with Parafilm and store at 4°C fridge overnight.

4 Day 4: Spinfection

4.1 Warm centrifuge to 30°C. Take out transfected Plat-Es (48 hour time point) and activated T cells (24 hour time point) from the incubator and transfer inside a tissue culture hood.

4.2 Filter the supernatant from transfected PlatEs using either a 10mL syringe + 0.45um filter or a 50mL vacuum filter Steri-flip with 0.45um filter). Filter and collect retroviral supernatant.

4.3 Take out Retronectin-coated 24-well plates from fridge, and aspirate inside a tissue culture hood to remove liquid.

4.4 Transfer activated T cells into 50 mL conicals. Place conicals in a magnetic separator to remove Dynabeads. Transfer T cells to fresh 50 mL conicals.

4.5 Add 60U/mL IL-2 per mL of T cells. Gently shake, do not vortex to mix.

4.6 Add 25uL Lipofectamine 2000 per 3mL viral supernatant. Incubate for 5mins.

4.7 Dispense 1mL viral supernatant to respective Retronectin-coated 24-well plates.

4.8 Add 1mL T cells to each well containing viral supernatant (1:1 T cell: retroviral supernatant ratio, final IL-2 concentration at 30U/mL).

4.9 Seal the plates with Parafilm. Centrifuge plates at 30°C for 1 hour at 2000rpm, no brake.

4.10 Remove parafilm and incubate at 37'C, 5% CO2 for 48 hours.

Day 6: Splitting T cells 1:1

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5.1 Pool cells into a T-75 or T-150 flask.

5.2 Add equal volume of pre-warmed complete IMDM media to each flask. Incubate at 37'C, 5% CO2 for 24 hours.

6 Day 7: Harvest transduced activated T cells for adoptive cell transfer

6.1 Take 300uL aliquot of transduced T cells and dispense into flow tubes. Take a 10uL aliquot to count cells and assess viability by Trypan blue staining.

6.2 Measure transduction efficiency by Flow cytometry using either biotinylated Tn-OTS8 tetramer for 237 CAR, WE CAR, and TNGK CAR or biotinylated Tn-MUC-1 tetramer for 5E5 CAR T cells

6.3 Count total T cells, spin down at 300xg for 5 minutes and resuspend to 25x10^6 cells/mL in HBSS. Place on ice. Inject 0.2mL (5x10^6 T cells) per mouse via i.p. injection.