Immuno WM Oncology · ciberonc Centro de Investigación Biomédica en Red Cáncer · Josep Carreras LEUKAEMIA Research Institute

EXPERIMENTAL PROTOCOL: CAR-T cell production

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Introduction

This protocol is for the generation of CAR-T cells from healthy PBMCs donors. PBMCs are obtained from buffy coats through Ficoll isolation, usually frozen, although it can also be done with fresh samples.

Reagents

- RPMI1640 + 10% heat-inactivated FBS + pen/strep + gluamine/glutamax
- OKT3 anti-CD3 MoAb (eBioscience 16-0037-85)
- CD28.2 anti-CD28 MoAb (BD Bioscience 555725)
- Sterile dH_2O
- Human IL-7, premium grade (Miltenyi 130-095-362)
- Human IL-15, premium grade (Miltenyi 130-095-764)
- CAR-encoding lentiviral vectors, titrated

Instruments and tools

- Non-treated 24-well plates
- 50-mL Falcon tubes
- 15-mL Falcon tubes
- Trypan Blue + Neuebauer chamber (or similar)
- Laminar flow hood
- Incubator
- Centrifuge
- Cytometer

Before start

We usually prepare 10µL working aliquots of IL-7 and IL-15 at 100 ng/µL. After thawing an aliquot, we keep it in the fridge for up to a week.

PROCEDURE

T cells expansion & transduction

The culture and expansion of primary T cells is done in 24-well plates.

Day 1: Stimulation with OKT3/CD28

1. Calculate the number of wells needed. Each well will have 1×10^6 PBMCs. If plating a standard experiment with 3 donors from frozen vials containing 5-7×10^6 PBMCs, prepare 5-6 wells for each donor (so, 18 wells total).
2. Each well will need **0.5 mL of OKT3 and anti-CD28.2 antibody solution**; calculate the total volume needed. Dilute the antibodies to a final concentration of 1 µg/mL in **sterile water** (1:1000 dilution for antibodies at 1 mg/mL). **Mix well**. i.e.: 18 wells × 0.5 mL/well = 9 mL total, plus 9 µL of each antibody.
3. Add 0.5 mL of the antibody solution per well in a 24-well plate. Make sure the solution entirely covers the surface. Incubate at **37 ºC for at least 2-4 hours**. Alternatively, the antibody solution can be added the previous day and incubated at 4 ºC overnight.
4. Aspirate the antibody solution and add **1 mL of complete media** (RPMI1640 + 10% FBS + Gln + p/s) to each well. Incubate for **15-30 min @ 37 ºC**. The purpose of this is to wash away the excess antibody and block remaining plastic w/ FBS.
5. In the meantime, **thaw the frozen PBMC vials**, transfer cells to a 15-mL tube containing about 10 mL of warm media. Centrifuge (400 g, 5 min), discard the supernatant to eliminate any residual DMSO, count cells, and resuspend them at **1×10^6 cells/mL** in complete media.
6. Aspirate the media from the OKT3/CD28 plates and add **1 mL of complete fresh media** plus the **1 mL of cells** on top. Therefore, each well will contain 1×10^6 PBMCs in 2 mL of media. Put the plate back in the incubator.

Day 2: Stimulation with IL-7/IL-15

1. Calculate the total volume of media in the plate. For example, 3 donors × 6 wells each × 2 mL per well = 36 mL.

2. Thaw IL-7 and IL-15 aliquots. Cytokines will be added in 50 uL of media to each well. Calculate the volume of media needed. 18 wells × 50 uL = 900 uL in our case. Prepare extra to compensate for pipetting errors.
3. Calculate the amount of cytokines needed given that they will be **diluted 1:10.000** (the working stock aliquots should be at 100 µg/mL) to a **final concentration of 10 ng/mL**. Add cytokines to an Eppendorf tube with the media. So, for the 36 mL we will need 3.6 µL of each cytokine. To account for pipetting errors, we can prepare 1000 µL media + 4 µL of each cytokine).
4. **Mix well** and **add 50 µL** of the cytokine cocktail into each well with activated PBMCs. Put the plate back in the incubator.

From here on, any fresh media that we add to the culture should contain IL-7 + IL-15.

Day 3: Lentiviral transduction

1. Resuspend thoroughly the cells in the wells with the p1000 pipette and collect them in a 15-mL Falcon tube.
2. Count the cells, centrifuge, and resuspend them in fresh complete media + cytokines at **1×10^6 cells/mL**.
3. Plate the cells into a new 24-well plate (without OKT3/CD28 coating). We usually plate **5×10^5 cells in 0.5 mL** in however many wells we need per donor. More is not always better; two wells of 5×10^5 cells each is better than one w/ 1×10^6 cells.
4. Add the required volume of the **lentiviral vector**(s) to achieve an **MOI of 10**. For 5×10^5 cells, we will need 5×10^6 transducing units. Plate additional untransduced wells as controls.

From here on, cells should be split every 2-3 days and kept at around 1×10^6 cells/mL in culture, always using complete media + cytokines. We prepare Falcon tubes w/ 50 mL of complete RPMI + 5 µL of IL-7 and IL-15 (100 ng/µL) each. It is preferable to not dilute the cells until day two after infection, to maximise the transduction efficiency. Transduction should be at least 20%.

Proper T cell activation and transduction should be assessed by flow cytometry checking **CD3**, **CD4**, **CD8**, **CD25** and **CD69** expression, as well as reporter genes or surface staining w/ **anti-F(ab)$_2$** (Jackson ImmunoResearch) or a **recombinant target protein** to detect the CAR construct (minimum 2 days after transduction).

T cells can be expanded for up to 12-14 days, but we usually plate assays at days 8-10.