TREG PURIFICATION, CULTURE AND TRANSDUCTION WITH RETROVIRAL VECTORS

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Treg have the unpleasant characteristic of proliferating less vigorously than conventional T cells (Tconv), at least in vitro. This leads to overgrowth of Tconv in culture if they minimally contaminate the initial Treg preparation, and to issues in getting decent numbers after expansion. This protocol minimizes both aspects and teaches how to transduce Treg effectively with retroviral vectors.

MATERIALS

ACK buffer to lyse red blood cells (NH_4Cl 150mM, $KHCO_3$ 10mM Na_2EDTA 0.1mM)
Miltenyi buffer (PBS 0.5% BSA 2mM EDTA)
Treg isolation kit (Miltenyi)
Treg culture medium:
BASE: DMEM with Glucose (4.5g/L), GlutaMax (1:200), HEPES(1:200) , non-essential aminoacids (1:100), sodium pyruvate (1:200). This base is aliquoted (45ml), sealed to prevent alkalinization, and either used or frozen.
FULL MEDIUM: Base + 5ml Treg-grade serum and 55μM β-mercaptoethanol (1:1000), use up or discard in 7 days.
Mouse IL-2 (R&D 1150-ML)
Anti-CD3/anti-CD28 coated beads (Invitrogen 114.56D)
mAbs to detect CD4, CD62L, Foxp3 by flow cytometry.

METHOD

Day 1:

1. Purify Treg:
    a. Sacrifice mice, take spleen and LN (axillary, brachial, inguinal).
    b. Smash organs in sterile conditions. Put them in a petri dish and keep wet with PBS. LNs can be ground using 2 frosted and ethanol-wiped glass slides (be careful not to break them). Filter the suspension (40μm filter) and thoroughly wash the Petri dish. Centrifuge the cells.
    c. Lyse red blood cells by resuspension in 1ml ACK for 5' at room temperature. Block lysis by adding 9ml PBS.
    c. Count cells, centrifuge 500g 3'.
    d. immunomagnetic enrichment of Treg:
        i. Resuspend cells in 1μl buffer/10^6 cells, add 0.25μl CD4 negative selection cocktail/10^6, incubate 10' 4C.
        ii. Do not wash. Add 0.75μl buffer /10^6 cells, 0.5μl of anti-biotin beads /10^6 cells, and 0.25μl anti-CD25 PE /10^6 cells. Incubate 15' 4C.
        iii. Wash with 5ml Miltenyi buffer, centrifuge 500g 3', resuspend in 0.5ml Miltenyi buffer.
        iv. Apply on LD columns (~$120x10^6$ cells per column) as described in step 1.d. Collect the negative fraction.
        v. **PRO TIP** Count cells, which should be ~20% of input. If it is much more, enrichment likely failed (kit expires exactly as advertised).
        vi. Centrifuge 500g 3'.

vii. Resuspend cells in 1μl anti-PE beads /10^6 cells, and 9μl of buffer /10^6 cells. Also add the other (non-PE labeled!) antibodies, e.g. CD4-PB 1:200, CD62L-APC 1:100. Incubate 15' 4C. Add 5ml Miltenyi buffer and centrifuge 500g 3'. Resuspend in 0.5ml Miltenyi buffer.

viii. Apply on MS column (100x10^6 cells per column).

- Prime columns with 0.5ml Miltenyi buffer
- Apply cells, resuspended in 0.5ml Miltenyi buffer
- Wash 3x with 0.5ml Miltenyi buffer.
- Keep positive fraction as $CD4^+CD25^+$ Treg and negative fraction as $CD4^+CD25^-$ responders.
- Centrifuge enriched Treg at 500g 3' and resuspend them in 700 μl T cell medium.

e. FACS sort Treg ($CD4^+CD25^{hi}CD62L^+$). If by any chance you had GFP expressed by the Foxp3 locus, sort Treg as $CD4^+GFP^+CD62L^+$. **NOTE** This step is essential to purify Tregs as much as possible. Even a small Tconv contamination will screw up the culture!

f. Carefully transfer to a 15ml Falcon tube and centrifuge 500g 3'.

g. Keeping the count given by the flow sorter, resuspend Treg at 1x10^6/ml in Treg medium.

2. Culture Treg:

a. Add IL-2 (400ng/ml, ~2000IU/ml) and CD3/CD28 coated beads, at a bead/cell ratio of 4:1.

b. Seed as follows:

<50000 : U bottom 96wells plate, 1 well.
50000 to 200000 : flat bottom 96wells plate, 1 well
200000 to 500000 : 48wells plate, 1 well (or 2-3 wells of 96wells plate)
500000 to one million: 24wells plate, 1 well. **NOTE** This is the most common condition, and the rest of the protocol is based on the usage of this vessel.

c. Spin the plate down 1' 500g to facilitate cell-bead contacts.

d. Incubate for 2 days. **NOTE** Do not touch them in the meanwhile!

Day 3:

1. Prepare retroviral vector by supplementing the retroviral supernatant with IL2 400ng/ml and Polybrene 4 μg/ml. Calculate using 500μl of mixture per well.
2. Apply retroviral supernatant on cells. **NOTE** The general idea is to avoid disturbing Tregs as much as possible.
    a. Spin the culture plate to make Tregs stick to the bottom, 1' 500g.
    b. Delicately transfer old supernatant to a Falcon tube, to recover cells that did not stick to the bottom of the well. Immediately apply 400μl of retroviral supernatant supplemented with IL2 and Polybrene.
    c. Spin the Falcon tube. Use the 100μl of mixture left to resuspend the cells in the Falcon tube, and add them to the respective well.
3. Proceed with spinfection: 1000g, 90' 32°C.
4. To replace retroviral supernatant with regular Treg medium + IL-2 400ng/ml, repeat the procedure at points 2b and 2c.

Day 4:

1. Perform second spinfection, identical to day 3, points 1, 2 and 3.
2. Remove the anti-CD3/anti-CD28 beads

a. Resuspend Tregs thoroughly and wash the wells, collect all in a 15ml Falcon tube (final volume ~5ml).
b. Put the tube in the Invitrogen Magnet and let sit 5'.
c. Without taking the tube out of the magnet, transfer the supernatant to a new tube.
d. Wash the original tube: take it out of the magnet and resuspend the beads accumulated on the sides with 5ml PBS. Put the tube back on the magnet for 5', then transfer the supernatant without taking the tube out of the magnet.
e. Spin the collection tube

3. Culture at $1x10^6$/ml in Treg medium + IL2 400ng/ml.

Day 6:

1. Count. Expect 10-20x expansion as compared to day 1.
2. Quality control:
    a. Flow cytometry of live cells to quantify transduction (typically 30 – 50%)
    b. Intranuclear flow cytometry for Foxp3. Must be >90% $Foxp3^+$, otherwise discard.
3. Use in experiments.