IN VITRO ASSAY TO EVALUATE TREG SUPPRESSION ON A PER CELL BASIS

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This protocol is a validated variation of the classic co-culture based test to evaluate suppressive activity of Treg. Key features include the usage of minimal amounts of cells, the separate evaluation of cTreg and eTreg and, in the analysis phase, the calculation of suppressive activity on a per cell basis.

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)
Anti-CD90.2 microbeads (Miltenyi)
Treg isolation kit (Miltenyi)
T cell culture medium: RPMI 10% FCS with HEPES(1:66) , non-essential aminoacids (1:100), sodium pyruvate (1:100), and β-mercaptoethanol (55μM)
CellTrace Violet
Anti-CD3 antibody: clone 145-2C11 from Biolegend.
mAbs to detect CD4, CD62L, CD44 and Foxp3 (PE strongly preferred) by flow cytometry.
Foxp3 fixation/permeabilization buffer set - eBiosciences

METHOD

Day 1:

1. Prepare T-depleted splenocytes (aka APC-ΔT):
    a. Sacrifice a WT mouse, collect the spleen and prepare single cells. Centrifuge 500g 3'.
    b. Lyse red blood cells by resuspending splenocytes in ACK buffer for 5' RT. Block lysis by adding 9ml PBS. Centrifuge 500g 3'.
    c. Resuspend all the splenocytes in 450μl Miltenyi buffer + 50μl of anti-CD90.2 beads, incubate 15' at 4C, add 5ml Miltenyi buffer, centrifuge 500g 3'.
    d. Run cells through a Miltenyi LD column:
        i. Prime LD columns with 2ml Miltenyi buffer
        ii. Apply cells in 0.5ml Miltenyi buffer
        iii. Wash column with 2 x 1ml Miltenyi buffer.
        iv. Collect negative fraction (flowthrough).
        v. Centrifuge 500g 3'.
    e. Resuspend these APC-ΔT in T cell culture medium at $4x10^5$/ml
    f. Keep in the fridge till assay is assembled.

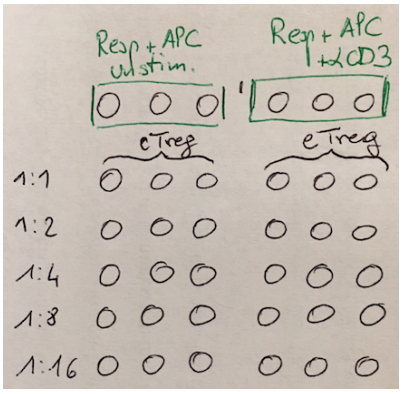
2. Prepare Treg and Responder cells:
    a. Sacrifice mice, usually at least one WT and one with the genetic deficiency to be tested.
    b. Prepare splenocytes and lyse red blood cells by resuspension in 1ml ACK for 5' at room temperature. Block lysis by adding 9ml PBS.
    c. Count cells, centrifuge 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, CD44 Ax700 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 cTreg ($CD4^+CD25^{hi}CD62L^+CD44^{lo}$) and eTreg ($CD4^+CD25^{hi}CD62L^-CD44^{hi}$). If by any chance you had GFP expressed by the Foxp3 locus, sort cTreg as $CD4^+GFP^+CD62L^+CD44^{lo}$ and eTreg as $CD4^+GFP^+CD62L^-CD44^{hi}$.
f. Carefully transfer to a 15ml Falcon tube and centrifuge 500g 3'.
g. Keeping the count given by the flow sorter, resuspend cTreg and eTreg at 2x10^5/ml in T cell culture medium. Keep in fridge until usage.

3. Label Responder cells with CellTrace Violet (CTV):
a. Resuspend all Responder cells obtained from step 2.d.vi in 1ml PBS 1%FCS.
b. Dilute CTV in PBS 1% FCS 1:500 (typically, 2μl for 1ml) to make a 2x solution.
c. Mix 1ml of cells with 1ml of diluted CTV and immediately vortex.
d. Incubate 20' at 37C.
e. Carefully pipette 5ml pure FCS under the cells and spin 5' 500g
f. Resuspend labeled responder cells at 2x10^5/ml in T cell culture medium. Keep in fridge until usage.

4. Compose the assay:



Typical setup for one kind of Responders and one of cTreg / eTreg in a U-bottom 96-well plate. One would need:

APC-ΔT: 7.2x10^5
Labeled responders: 3.6x10^5
cTreg / eTreg: 6x10^4 each

a. Dilute anti-CD3 mAbs in T cell culture medium at the concentration of 1μg/ml (4x).
b. In a U-bottom 96-well plate, start plating c and eTregs.
   i. Put 100 μl of Treg cell suspension in all the wells corresponding to the 1:1 Responder:Treg ratio.
   ii. Pipette 50 μl of T cell culture medium in all other wells.
   iii. Using a multichannel pipette, take 50ul of Treg from the 1:1 wells and put them in the 1:2 wells, pipette well, take 50 μl of Treg from the 1:2 wells and put it in the 1:4 wells, and so on so forth until you get to the 1:16 dilution. You have 100μl there, discard 50 μl of it. You should end up with serially diluted Treg from $10x10^3$ per well to 625 per well, all wells having 50 μl of Treg suspension.
c. Plate APC-ΔT 50 μl per well, in all wells. It makes $20x10^3$ APC-ΔT per well.
d. Plate Responders 50 μl per well, in all wells. It makes $10x10^3$ Responders per well.
e. Plate diluted anti-CD3 mAbs, 50 μl per well. The final concentration of anti-CD3 antibody is 250ng/ml. This goes in all wells except the ones corresponding to the non-stimulated cells, where 50 μl of T cell medium should be put.
f. Incubate at 37C for 72h.

Day 4:

1. Transfer cells from the U-bottom plate to a V-bottom plate. Centrifuge 500g 3'.
2. Stain with 50 μl ZombieRed (1:400) diluted in PBS, incubate 15' 4C
3. Block with FcBlock (1:50) in 50 μl T cell medium, add on top of ZombieRed, incubate 10' 4C.
4. Surface staining: Prepare 100 μl of T cell medium with Abs 2x, add on top and incubate 15' 4C. Use the same fluorophore as for staining in day 1, step 2.d.vii. for CD4 (in this example, PB), add if relevant CD45.1 and CD45.2 or other congenic markers.
5. Centrifuge 500g 3'.
6. If Treg have GFP, one may resuspend in 200 μl PBS 0.5% BSA and go to step 10. Otherwise, do the following:
7. Fix cells using the Foxp3 fixation/permeabilization buffer (to be diluted 1:4 with buffer diluent immediately before use). Budget 200 μl per well. Incubate 30' RT. Centrifuge 800g 3'.
8. Dilute Foxp3-PE in Foxp3 wash buffer (1:100) calculating 50 μl/well, and stain cells for 30' 4C.
9. Add 150μl Foxp3 wash buffer, centrifuge 800g 3' and resuspend cells in 200 μl Foxp3 wash buffer. Go to the cytometer.
10. Set the cytometer to acquire ALL the sample in the well, in order to be able to count how many Treg you have in each well.

Any day after 4:

1. Calculate Fold Increase (FI) of responders using the CTV dilution plot.
   a. Draw linear gates corresponding to every peak of the CTV, and count cells in each.
   b. Calculate the number of progenitors that gave rise to the plot. For cells that divided 0 times it is all of the cells, for cells that divided once it is the cell number / 2, for cells that divided 2 times it is the cell number / 4, and so on. Sum all progenitors together. In mathematical terms, this is:

$$progenitors = \sum_{i=0}^{n} \frac{count_i}{2^i}$$

where n is the maximum number of divisions.

c. Calculate the FI as count(all Responders)/progenitors.

2. Scale FI into % of suppression considering that Responders w/o Treg represent 0% suppression, and cells that have not proliferated represent 100% suppression.
3. Relate % suppression to the amount of counted Treg.