PBMC-MSC Co-cultures for Induction of Treg Generation
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[Abstract] To assess the capacity of multipotent stromal cells (MSC) to induce the generation of Tregs, transwell co-cultures were performed as well as cultures with MSC-conditioned medium (CM). In short, peripheral blood mononuclear cells (PBMC) were co-cultured with allogeneic MSC or CM for one week followed by one week of culture in the absence of MSC.

Materials and reagents

1. Peripheral blood mononuclear cells (PBMC) [isolated from a healthy donor using Ficoll-Paque (own pharmacy) density gradient (1.077 g/cm³)]
2. Multipotent stromal cells (MSC) from healthy donors
3. Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, catalog number: 31870-082)
4. Penicillin/streptomycin (5,000 U/ml) (Life Technologies, catalog number: 15070-063)
5. L-glutamin (200 mM) (Life Technologies, catalog number: 25030-024)
6. Fetal calf serum (FCS) (Greiner Bio-One GmbH)
7. Phosphate buffered saline (PBS)
8. Trypsin/EDTA (1x 0.05% Trypsin-EDTA, phenol red) (Life Technologies, catalog number: 25300-096)

Equipment

1. T75 culture flasks
2. 12-well transwell plates (pore size 0.4 μM) (Sigma-Aldrich, catalog number: CLS3460)
3. 10 K Centriprep Centrifugal filters (Millipore, catalog number: 4304)
4. 12/24/48-well plates (Sigma-Aldrich, Corning Costar cell culture plates)
5. 37 °C, 5% CO₂ cell culture incubator
6. Microscope
7. Centrifuge
8. 10 K Centriprep centrifugal filters
9. Hemocytometer (counting chamber) or Sysmex F-820
Procedure

A. Harvesting and counting of MSC
   1. Wash the cells once with PBS to remove culture medium.
   2. Incubate for 5 min with trypsin/EDTA.
   3. Check under the microscope whether MSC are detached.
   4. Harvest cells in culture medium diluted 5x in PBS.
   5. Spin down at 350 \( \times g \) and resuspend cells in 1-3 ml of culture medium.
   6. Count cells using a hemocytometer.

B. PBMC-MSC co-culture in transwell system
   Culture medium: RPMI medium + 10% FCS + P/S (100 U/ml) + L-glutamin (100 U/ml). L-glutamin is added freshly at day 0.
   1. At day 0 100,000 MSC are plated in the lower well of a 12-wells transwell plate in 1.5 ml culture medium.
   2. 400,000 PBMC are added in the transwell insert in 500 \( \mu l \) of culture medium.
   3. Co-cultures are cultured for one week at 37 °C in a 5% CO\(_2\) cell culture incubator.
   4. At day 7, PBMC are collected from the inserts.
   5. Spin down PBMC at 350 \( \times g \) for 10 min.
   6. Add 120 \( \mu l \) of PBS and count PBMC with sysmex or hemocytometer.
   7. Calculate total number of PBMC per condition and replate the PBMC in fresh culture medium and fresh plates:
      a. 350,000-700,000 PBMC: 24-wells in 1 ml medium
      b. < 350,000 PBMC: 48-wells in 750 \( \mu l \) medium
      *Note: Plating too low numbers of cells will result in bad Treg generation.*
   8. Co-cultures are cultured for one week at 37 °C in a 5% CO\(_2\) cell culture incubator.
   9. At day 14, collect PBMC.
   10. Spin down PBMC 350 \( \times g \) for 10 min.
   11. Add 120 \( \mu l \) of PBS and count PBMC with sysmex or hemocytometer.
   12. Further analyze PBMC with flowcytometry.

C. MSC-PBMC co-culture with MSC conditioned medium (CM)
   1. Let MSC grow to near-confluence in a T75 flask.
   2. Change the medium with culture medium and culture for 7 days without changing the medium.
   3. Collect the culture medium after 7 days (= day 0), this the MSC conditioned medium (CM).
4. Harvest and count the MSC.
5. Spin down the CM 350 x g for 10 min to get rid of cell debris.
6. Concentrate the cell free CM using 10 K Centriprep centrifugal filters (according to manufacturers’ instructions).
   a. Wash the Centriprep centrifugal filters with 10 ml PBS; spin down 3,000 x g 10 min.
   b. Add MSC-CM (14 ml) and spin down for 30 min at 3,000 x g.
   c. Collect concentrated CM and calculate the volume of CM that is equivalent to 100,000 MSC.
      i. Example: In step C5 1.0 x 10^6 MSC are counted and 25 ml CM is collected in step C4.
      ii. After concentration the CM has a volume of 5 ml (= 5 times concentrated).
      iii. 5 ml of concentrated CM is equivalent to 1.0 x 10^6 MSC, so 500 µl of concentrated CM is equivalent to 100,000 MSC.
7. Plate 400,000 PBMC in CM that is equivalent to 100,000 MSC and add 1 ml of fresh culture medium in a 12-wells plate.
8. Co-cultures are cultured for one week at 37 °C in a 5% CO2 cell culture incubator.
9. At day 7, PBMC are collected.
10. From this point onwards, continue as described in the co-culture in a transwell system starting at step C4.

References