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/cm^3)]
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% CO_2 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 *x 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 µl 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 *x g* for 10 min.
   6. Add 120 µl 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 µl 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 *x g* for 10 min.
   11. Add 120 µl 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% CO_2 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

1. Melief, S. M., Schrama, E., Brugman, M. H., Tiemessen, M. M., Hoogduijn, M. J., Fibbe, W. E. and Roelofs, H. (2013). Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. *Stem Cells* 31(9): 1980-1991.

