Chemotaxis Migration Protocol for Macrophages

Materials

- Freshly isolated blood (15 mLs)
- RosetteSep Human Monocytes Enrichment Cocktail (Stem Cell Technologies 15028)
- Lymphoprep (Stem Cell Technologies 07801)
- RPMI 1640 Medium (Life Technologies 11875-085)
- Fetal Bovine Serum (Sigma-Aldrich F2442-500mL)
- EDTA (Ambion/Invitrogen AM9260G)
- Recombinant Human GM-CSF for M1 differentiation (Peprotech 300-03)
- Recombinant Human M-CSF for M2 differentiation (Peprotech 300-25)
- LPS for M1 differentiation (Sigma-Aldrich L4391-1MG)
- Recombinant Human IFN-γ for M1 differentiation (Peprotech 300-02A)
- Recombinant Human IL-4 for M2 differentiation (Peprotech 200-04)
- D-PBS (w/o Ca^{2+}, Mg^{2+}, Life Technologies 10010)
- StemPro® Accutase® (Life Technologies A111-05-01)
- Matrigel® Matrix (Corning 354234)
- Recombinant Human C5a (Peprotech 300-70)
- IncuCyte™ ClearView™ 96-Well Cell Migration Plate (Essen 4582 or 4599)

Isolate PBMCs

1) Prior to beginning isolation, ensure that medium, RosetteSep and blood are all at ambient temperature.
2) Add EDTA to whole blood to a final concentration of 1 mM (e.g. add 10 µL of a 100 mM solution of EDTA per 1 mL of whole blood).
   NOTE: This step if not necessary if using EDTA-coated tubes for blood isolation.
3) Add RosetteSep™ Enrichment Cocktail at 50 µL/mL of whole blood (e.g. for 15 mL of whole blood, add 750 µL of cocktail). Mix well.
4) Incubate 20 minutes at room temperature (15 - 25°C).
5) Dilute sample with an equal volume of RPMI 1640 and mix gently.
6) Layer the diluted sample on top of the Lymphoprep medium OR layer the Lymphoprep underneath the diluted sample (e.g. 15 mLs of blood + 15 mLs of medium + layer 15 mLs of Lymphoprep in a 50 mL conical tube).
   NOTE: Be careful to minimize mixing of the density gradient medium and sample.
7) Centrifuge for 20 minutes at 1200 x g at room temperature (15 - 25°C), with the brake off.
8) Remove the enriched cells from the density gradient medium:plasma interface.
   NOTE: Sometimes it is difficult to see the cells at the interface, especially when very rare cells are enriched. It is advisable to remove some of the density gradient medium along with the enriched cells in order to ensure their complete recovery.
9) Wash enriched cells with 10 mL of RPMI 1640 + 10% FBS medium. Repeat wash.
10) After second wash resuspend the cells with the appropriate medium in order to differentiate cells into M1 or M2 (outlined below).

Differentiation of Macrophages

1) 1) Replace the media with RPMI + 10% FBS + 50 ng/mL GM-CSF (M1) or 100 ng/mL M-CSF (M2) and perform a cell count.
2) Seed a T25 flask with about 400,000 cells. Alternatively, cells can be maturated directly within a Matrigel® coated ClearView™ plate (coated with Matrigel® 5 µg/mL in 10% FBS) at 3K/well.
   NOTE: If differentiating within the ClearView™ plate, 200 µL of PBS should be placed in the reservoir at the time of cell seeding.
3) Allow cells to differentiate for 6 days. Change media every 3-4 days or if it turns yellow. At day 6, change media to RPMI + 10% FBS + 50 ng/mL GM-CSF + 1 ng/mL LPS + 20 ng/mL IFN-γ (M1) or RPMI + 10% FBS + 20 ng/mL IL-4 + 100 ng/mL M-CSF (M2) for about 24 hours.

Chemotaxis Assay

1) Coat both sides of the membrane with 50 µg/mL Matrigel® + 10% FBS, adding 20 µL to the insert wells (reverse pipette) and 150 µL to the reservoir wells (pre-fill reservoir and gently place the insert into the reservoir plate containing coating matrix). In this case, a second reservoir plate will be loaded with chemoattractant and used during the experiment.
   NOTE: The cell migration plate and all reagents must be pre-chilled to 4°C. We recommend using a CoolSink™ plate to keep the plate cold during the coating procedure.
2) Place the ClearView™ cell migration plate at 37°C and incubate for 30 minutes.
3) Remove the ClearView™ plate from 37°C and allow it to cool to ambient temperature for 30 minutes.
   NOTE: This step is important in order to achieve uniform cell distribution within each well.
4) Immediately prior to macrophage addition, aspirate Matrigel® Matrix coating from both the reservoir plate and insert. Add 200 µL D-PBS to the reservoir plate then gently place the insert into the reservoir.
5) Harvest differentiated Macrophages using Accutase and perform a cell count (e.g., trypan blue staining + hemacytometer). Centrifuge the cell suspension (350 x g, 4 minutes) and resuspend the cell pellet in RPMI1640 + 0.5% FBS at 33,333 cells per mL.
6) Using a manual multi-channel pipette and reverse pipetting technique, seed cells (60 µL per well, 2,000 cells per well) into every well of the insert plate.
   Calculation:
   33,333 cells/mL x 0.06 mL = 2,000 cells per insert well.
7) Allow macrophages to settle on the membrane at ambient temperature for 15-30 minutes.
8) Using a manual multi-channel pipette, add 200 µL of the chemoattractant (for Macrophages we recommend C5a as a positive control) and control medium to the appropriate wells of the second reservoir plate.
9) Carefully transfer the insert plate containing the cells into the pre-filled second reservoir plate containing medium ± chemoattractant.
10) Place the IncuCyte™ ClearView™ cell migration plate into the IncuCyte ZOOM® instrument and allow the plate to warm to 37°C for at least 15 minutes. **After 15 minutes, wipe away any condensation that remains on the outside of the plate lid or bottom of the reservoir. At this point, the IncuCyte ZOOM drawer should remain closed to prevent lightly adhered macrophages from falling off of the bottom side of the membrane.**
11) In the IncuCyte ZOOM® software, schedule 24 hour repeat scanning (10x) for every 30 minutes.
    NOTE: This schedule is only for a scanning a single plate. Fewer scans times will be required if scheduling multiple plates
    a. Objective: Ensure 10x objective is installed
    b. Vessel Type: Select “ClearView Cell Migration”
    c. Channel Selection: Select “Phase”
    d. Scan Mode: Select “Chemotaxis (Top/Bot)” scan type and desired Scan Pattern

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