Macrophage 1/Macrophage 2.

Procedure:

- Obtain appropriate amount of Leucosept tubes (3-4 per buffy coat). Regular 50ml Falcon can also be used of course.
- Add 15 ml of ficoll to each tube (for Leucosept tubes spin 1 minutes at 1000 rpm)
- Dilute buffy (50mls approx 100x10^6 PBMC) 1 in 2 with PBS++ add equal volume to each (Leucosept) tube.
- Centrifuge the tubes for 20 minutes at 2000 rpm in a Beckman swing out rotor (without brake if not using leucosept tubes)
- Place the interphase into a new 50 ml tube (pool 2 Leucosept tubes to 1 50 ml tube) don't bother with the fat and cells attached to the side of tubes.
- Fill up the tubes (containing the cells) with rt PBS to a volume of 50 ml and invert 3 times
- Centrifuge the tubes for 10minutes at 1800 rpm in a Beckman swing out rotor with low brake (this step removes residual ficoll)
- Gently pour of supernatant and resuspend carefully to not activate monocytes
- Fill up the tubes with PBS to a volume of 50 ml and invert 3 times
- Centrifuge the tubes for 10minutes at 1000 rpm in a Beckman swing out rotor with low brake (this will remove most platelets from your pellet)
- Gently pour of supernatant and resuspend by gentle pipetting
- Fill up the tubes with PBS to a volume of 50 ml and invert 3 times
- Count the cells.
- Centrifuge the tubes for 10 minutes at 1000 rpm in a Beckman swing out rotor with low brake (removes residual platelets)
- Gently pour off supernatant and resuspend in appropriate volume of MACS buffer (PBS containing 2 % FCS and 2mM EDTA) using a 1 ml pipet (80 µl Macs buffer to $5 \bullet 10^7$ cells)
- Add CD14 Beads and mix gently (20 µl CD14 beads to $5 \bullet 10^7$ cells)
- Incubate the cell-bead mixture for 15 minutes at 4°C (NOT ICE) (keep a beaker of water in the fridge and submerge your tubes to reach optimal temperature rapidly)
- Fill up the cell-bead mixture to 50 ml with PBS and invert 3 times
- Centrifuge the tubes for 10 minutes at 1500 rpm in a Beckman swing out rotor with low brake
- Completely remove the supernatant
- Carefully resuspend the cells in an appropriate MACS buffer (500 µl Macs buffer to 10^8 cells)
- Apply cells to the humidified column.
- Wash the cells with three times 5 ml MACS buffer (try to keep a continues flow)
- Transfer the LS+ column (containing cells) to a 15 ml tube and add 5 ml of MACS buffer
- Use the plunger to force out the CD14+ cells
- Count the CD14+ cells while centrifuging the tubes for 10 minutes at 1500 rpm in a Beckman swing out rotor with low brake
    - *check by FACS !*

- Plate out the cells at a density of at least $1 \bullet 10^6$ cells/ml in RPMI supplemented with 10% FCS (Greiner)* and L-glutamine (Gibco) and containing the appropriate differentiation factors (concentrations can vary with batches !). 10 ml of medium with cells in a T75 Flask
- Incubate the cells at 37°C and 5% CO_2 for three days.
- At day 3 at 0.5 volumes extra medium to each flask containing the differentiation factors.
- Incubate the cells at 37°C and 5% CO_2 for three more days.
- At day 6 the cells are ready to be harvested and used in subsequent experiments.
- Trypsinize cells and replate overnight prior to experiments. Usual densities regularly used: 300 000 cells/well/24 well plate or 30 000 cells/well/96 well plate. Persevere with the mφ1 cells as they can be very, very, very adherent. Carefully but firmly whacking the flasks against a table in the plane of the flask culture surface usually helps to dislocate the cells after 5 mins trypsin..

- **Quality Control on Macrophages:**

- Seed macrophages at 3x 30.000 cells per well in a 96 well flat bottom plate.
- Adjust volume to 100 µl/well for 24 hours at 37
- FACS cells for CD14 CD163, HLA-DR, CD80 and CD86 (preferably before and after LPS stimulation if there re enough cells)
- Add 50 µl LPS(e.coli) at 300 ng/ml, (Final concentration is then 100 ng/ml)
- Incubate for 24 hours at 37°C , 5% CO_2
    - also take an un-stimulated sample
- Measure IL-12p40 and IL-10 levels!